

## BEST AVAILABLE COPY

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>C12Q 1/68, G01N 27/26</b>		A1	(11) International Publication Number: <b>WO 95/07363</b>  (43) International Publication Date: <b>16 March 1995 (16.03.95)</b>
(21) International Application Number: <b>PCT/US94/09764</b> (22) International Filing Date: <b>8 September 1994 (08.09.94)</b>		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 08/120,006 10 September 1993 (10.09.93) US		<b>Published</b> <i>With international search report.</i>	
(60) Parent Application or Grant (63) Related by Continuation US 08/120,006 (CIP) Filed on 10 September 1993 (10.09.93)			
(71) Applicant ( <i>for all designated States except US</i> ): GENEVUE, INC. [US/US]; 1199 Camino Vallecito, Lafayette, CA 94549 (US).			
(72) Inventor; and (75) Inventor/Applicant ( <i>for US only</i> ): KONRAD, Michael, W. [US/US]; 1199 Camino Vallecito, Lafayette, CA 94549 (US).			
(74) Agents: TERLIZZI, Laura et al.; Skjerven, Morrill, MacPherson, Franklin & Friel, Suite 700, 25 Metro Drive, San Jose, CA 95110 (US).			

(54) Title: OPTICAL DETECTION OF POSITION OF OLIGONUCLEOTIDES ON LARGE DNA MOLECULES

## (57) Abstract

A method for analyzing a sample oligonucleotide sequence is described. The method comprises contacting the sample oligonucleotide sequence with an anchor sequence which comprises an immobilized oligonucleotide sequence which hybridizes with the sample. The sample is also contacted with a probe comprising an oligonucleotide sequence which hybridizes to a target oligonucleotide sequence to be detected in a suitable buffer to form a complex. The complex is subjected to a field which moves unbound oligonucleotide sequences away from the anchor sequence in the direction of the field, and preferably, extends the sample sequence. Whether the probe is bound to the sample oligonucleotide sequence, and preferably, the position of the probe, is determined to determine whether the target oligonucleotide sequence is present in the sample. The method can be used for mapping, for identity typing, and to determine whether a test oligonucleotide sequence is present in the sample. A device for performing the method and reagents are also described.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

OPTICAL DETECTION OF POSITION OF  
OLIGONUCLEOTIDES ON LARGE DNA MOLECULES

5

Field of the Invention

The present invention relates to analysis of DNA sequences and, in particular, to optically determining the position of an oligonucleotide sequence on a large 10 DNA molecule.

Background of the Invention

There have been significant advances in DNA analysis methods in recent years. At present, there 15 are several distinct methods for DNA analysis. There are several methods which involve use of an oligonucleotide sequence as a probe to bind to a complementary oligonucleotide sequence which may be present in the sample. This method takes many 20 embodiments from fluorescent in situ hybridization of chromosomes in tissue sections to dot blot analysis of DNA fragments.

There are several DNA analysis methods which involve amplification, usually using the polymerase chain 25 reaction method. In that method, primer, usually on the order of 20 to 30 nucleotides, are used to amplify a region of DNA as large as a few kilobases. The amplified region can be analyzed by a number of methods, including use of probes or sequencing. 30 Alternatively, analyses can be based on the pattern of fragments produced in the amplification.

In addition, DNA can be digested with a restriction endonuclease and the fragment patterns produced can be analyzed to determine alleles present 35 in the sample DNA. Numerous additional methods are used in mapping and cloning to try to identify the location of a gene or to determine which variant of a known gene is present in a sample. All of these

analysis methods have advantages and disadvantages and additional, more informative methods are still being sought.

##### 5    Summary of the Invention

The present invention provides a method for analyzing a sample oligonucleotide sequence. The method comprises contacting the sample oligonucleotide sequence with an anchor sequence which comprises an 10 oligonucleotide sequence which is immobilized to a support and which hybridizes with the sample oligonucleotide sequence. The sample oligonucleotide sequence is also contacted with a probe comprising an oligonucleotide sequence which hybridizes to a target 15 oligonucleotide sequence to be detected. The contacting is performed in a suitable buffer to form a complex. The resultant complex is subjected to a field which moves unbound oligonucleotide sequences away from the anchor sequence in the direction of the field. 20 Whether the probe is bound to the sample oligonucleotide sequence is determined to determine whether the target oligonucleotide sequence is present in the sample oligonucleotide sequence. Preferably, the complex is subjected to the field for a time 25 sufficient to extend the sample oligonucleotide sequence and the position of the probe in relation to the anchor sequence is also determined.

The field can be a magnetic or, preferably, an electric field in which the oligonucleotide sequences 30 migrate based on their inherent charges. Preferably, the probe is labeled with a fluorochrome, preferably a fluorochrome which is present in a bead, which facilitates use of a fluorescent microscope to determine the presence or position of the probe on the 35 sample oligonucleotide sequence.

In one embodiment, the method is used for mapping.

The method can determine whether a first and a second target are on a molecule of sample DNA by having the anchor sequence hybridize to the first oligonucleotide sequence and the probe hybridize to the second

5        oligonucleotide sequence. Alternatively, the nucleotide sequence near the end of the sample DNA molecule to be mapped can be determined using short probes of random sequences, identifying a pair of probes which hybridize toward the end of the sample DNA

10      molecule, and using one probe and the complement of the other to amplify the segment between the probes by the polymerase chain reaction. The nucleotide sequence of this segment can then be determined, and one of the strands from this amplified segment can then be used as

15      a new anchor sequence, to walk down the DNA molecule.

In another embodiment, sample DNA is characterized (fingerprinted) using an anchor sequence which hybridizes to a conserved region in the sample DNA and a plurality of probes of random or arbitrary sequence

20      of from about 5 to about 15 bases. Following hybridization, the sample DNA is extended and unbound probes are removed by the field. The positions of the probes in relation to the anchor sequence are determined. The method can be used in paternity and

25      other forensic applications by using the same anchor and probes on the mother and putative father's DNA or crime scene and suspect DNA.

In another embodiment, the method can be used to determine whether a test oligonucleotide sequence is

30      present in the sample oligonucleotide sequence. In that embodiment, the anchor sequence comprises an immobilized, conserved oligonucleotide sequence known to hybridize with an oligonucleotide sequence in the sample oligonucleotide sequence and the probe

35      hybridizes with the test oligonucleotide sequence. The test oligonucleotide sequence can be any

oligonucleotide sequence of interest, such as, for example, a sequence characteristic of a disease gene allele. In a preferred embodiment, a plurality of different probes which bind to different test sequences 5 are used. Each of the probes is labeled with a different fluorochrome or combination of fluorochromes.

A device for performing the method and reagents are also described.

10    Brief Description of the Drawings

Figure 1 illustrates a top view of one embodiment of a DNA analysis device of this invention, prior to attachment of a cover.

15    Figure 2 illustrates a side view of another embodiment of a DNA analysis device of this invention having a composite electrode.

Figure 3 illustrates a top view of another embodiment of a DNA analysis device of this invention.

20    Figure 4 illustrates a side view of the embodiment of the device illustrated in Figure 3.

Description of the Invention

The present invention provides a method of analyzing oligonucleotide sequences in which the sample 25 oligonucleotide sequence is contacted with an anchor sequence and a probe in a suitable buffer for hybridization. The anchor sequence is an oligonucleotide sequence immobilized to a support which hybridizes with a conserved oligonucleotide sequence 30 present in the sample oligonucleotide sequence. The probe can be any oligonucleotide sequence of interest depending on the type of analysis to be performed. Following formation of a complex by hybridization of the sample oligonucleotide sequence to the anchor 35 sequence, the oligonucleotide sequences are subjected to a field which moves unbound probes away from the

anchor sequence. The field preferably also extends the complexed sample oligonucleotide sequence in the direction of the field, thereby facilitating determining the distance between the anchor sequence and the probe and thus the relative positions of the sequences to which the probe and anchor sequence bind. In a preferred embodiment, the probes are labeled with a fluorochrome, facilitating determining distances to within about forty nucleotides using a fluorescent microscope.

In one embodiment, the method can be used for mapping by determining whether two oligonucleotide sequences are on the same molecule of DNA and the distance and direction of the sequences. In another embodiment, the method can be used to characterize DNA sequences and to determine either whether sample and test DNA are from the same individual or whether a putative father can be the father of a child. In another embodiment, sample DNA can be analyzed to determine whether one or a plurality of oligonucleotide sequences, such as oligonucleotide sequences characteristic of a disease gene, are present. A device and reagents facilitating the method are also described.

25

Reagents and Device for Practicing the Method

The reagents and device used to practice the method of this invention are described below, followed by a description of the method.

30

The Sample Oligonucleotide Sequence

The sample oligonucleotide sequence can be any oligonucleotide sequence. The present invention is particularly advantageous in analyzing long oligonucleotide sequences; e.g., greater than sizes which can be readily analyzed by polymerase chain

reaction methods (2-3 kilobases). The sample oligonucleotide sequence can be DNA or RNA and can be single-stranded or double-stranded. Conveniently, the sample is a single-stranded oligonucleotide sequence, 5 preferably a DNA sequence. However, the method can be performed wherein either the sample oligonucleotide sequence or the anchor sequence is double-stranded and the complex formed by hybridization is a triple helix. The binding rules for triple helix formation are well 10 known and are described in International Application No. PCT/US89/05769 (Publication No. WO 90/06934, published June 28, 1990) to Hogan et al., for example. That application is incorporated herein by reference in its entirety.

15 Extraction of sample oligonucleotide sequences from cells and preparation of single-stranded oligonucleotide sequences are well known. All nucleated cells contain genomic DNA and RNA and, therefore, are potential sources of the sample 20 oligonucleotide sequence. For higher animals, peripheral blood cells are typically used rather than tissue samples. As little as 0.01 to 0.05 cc of peripheral blood provides sufficient DNA for analysis. Hair, semen and tissue can also be used as samples. In 25 the case of fetal analyses, placental cells or fetal cells present in amniotic fluid can be used. When the sample oligonucleotide sequence is DNA, the DNA is isolated from nucleated cells under conditions that minimize DNA degradation. Typically, the isolation 30 involves digesting the cells with a protease that does not attack DNA at a temperature and pH that reduces the likelihood of DNase activity. For peripheral blood cells, lysing the cells with a hypotonic solution (water) is sufficient to release the DNA. Generally, 35 red white blood cells which do not contain nuclei are separated from the nucleated white blood cells prior to

extraction of DNA.

DNA isolation from nucleated cells is described by Kan et al., *N. Engl. J. Med.* 297:1080-1084 (1977); Kan et al., *Nature* 251:392-392 (1974); Kan et al., *PNAS* 75:5631-5635 (1978); and in Sections 2.1 and 2.2 of Ausubel, *Short Protocols in Molecular Biology*, Second Edition, John Wiley and Sons, New York (1992). In addition, commercial DNA purification kits are available. (See page 190-191 in BioRad Laboratories: BioRad Laboratories Catalog: Life Science Research Products. Hercules, CA, Bio-Rad Laboratories (1993) which describes the InstaGene DNA purification kit.) Each of the above references is incorporated herein by reference in its entirety. Extraction procedures for samples such as blood, semen, hair follicles, semen, mucous membrane epithelium and other sources of genomic DNA are well known. For plant cells, digestion of the cells with cellulase releases DNA. Thereafter DNA is purified as described above.

Procedures for isolating RNA from cells are described in Sections 4.1 and 4.2 of Ausubel, *Short Protocols in Molecular Biology*, Second Edition, John Wiley and Sons, New York (1992) and pages 38 to 50 of Pharmacia Corporation: Pharmacia Catalog: Molecular and Cell Biology, Pharmacia Corporation, 1993.

The extracted oligonucleotide sequence can be purified by dialysis, chromatography, or other known methods for purifying oligonucleotides prior to analysis. Typically, the oligonucleotide sequence is not purified prior to analysis by the methods of this invention. However, usually, RNA in the sample is digested when the sample oligonucleotide sequence is DNA, and DNA is digested when the sample oligonucleotide sequence is RNA.

The method can be performed on a sample oligonucleotide sequence which is less than about 200

kb using normal handling techniques in preparing the sample oligonucleotide sequence. However, when the sample oligonucleotide sequence is greater than about the size of T4 DNA (greater than about 200 kb), special 5 handling techniques are used in preparing the sample oligonucleotide sequence.

The techniques for handling long oligonucleotide sequences are well known and involve processing techniques which minimize forces which can shear 10 oligonucleotide sequences. For example, cells containing the sample oligonucleotide sequence can be lysed in a gel to preserve the integrity of the oligonucleotide sequences. The gel is usually a gel which is solid at room temperature and liquid at an 15 elevated temperature such as 37°C. For example, on page 84 in Promega Corporation: Catalogue: Biological Research Products, Madison, WI, Promega Corporation, (1992-3) describes preparation of intact yeast chromosomes from whole cells embedded in 0.8% low melt point agarose. The cells are lysed and deproteinized 20 in situ as described in McClelland et al., *Nucl. Acid Res.* 15:5985 (1987).

A fragment of the gel is removed for analysis to avoid pipetting, mixing, and other techniques which 25 exert shear forces. The reagents for the analysis method are allowed to diffuse into the gel to achieve the desired effects on the cells containing the DNA or the DNA itself. When using such samples, the analysis procedure is performed at an elevated temperature at 30 which the gel is liquid.

When the sample oligonucleotide sequence is DNA, preferably, the DNA strands are separated into single stranded DNA prior to analysis. This strand separation can be accomplished by a number of methods including 35 physical or chemical means. A preferred method is the physical method of separating the strands by heating

the DNA until it is substantially (approximately 93%) denatured. Heat denaturation involves temperatures ranging from about 80° to 105°C for times ranging from about 15 to 30 seconds. Typically, heating the DNA to 5 a temperature of from 90° to 93°C for about 30 seconds to about 1 minute is sufficient. Alternatively, sample oligonucleotide sequences can be denatured by heating at 75°C for 5 minutes in 70% formamide (final concentration), 0.3 M NaCl, 0.01 M Tris-HCl, pH 8.5, 10 0.001 M EDTA.

The Anchor Sequence

The anchor sequence is an oligonucleotide sequence immobilized to a support which hybridizes with a 15 conserved oligonucleotide sequence present in the sample oligonucleotide sequence. The anchor sequence is also unique in the total oligonucleotide population the sample oligonucleotide has been obtained from; e.g. human genomic DNA. The anchor sequence can be any 20 oligonucleotide sequence which hybridizes to the sample sequence, forming a complex which is sufficient to immobilize the sample oligonucleotide sequence in the presence of an electric field that removes unbound probes. Preferably, the anchor sequence forms a 25 complex which is sufficient to immobilize the sample oligonucleotide sequence in the presence of an electric or magnetic field that extends or elongates the sample oligonucleotide sequence in the direction of the field, thereby facilitating determining the distance of the 30 probe from the anchor sequence.

The anchor sequence can be a DNA sequence or an RNA sequence. The anchor sequence is preferably a single-stranded oligonucleotide sequence to provide sufficient binding to immobilize the sample 35 oligonucleotide sequence in the electric field. However, double-stranded oligonucleotide sequence which

hybridizes to a single-stranded sample oligonucleotide sequence to form a triple helix are also contemplated. In addition, the use of a peptide nucleic acid oligomer sequence which can displace one strand of a double 5 helix to hybridize to the sequence on the second strand is contemplated (see Peffer et al., *Proc. Natl. Acad. Sci. USA* 90:10648-10652 (1993)).

The anchor sequence is preferably a conserved oligonucleotide sequence to ensure that the sample 10 sequence contains a sequence which hybridizes to the anchor sequence. In addition, the anchor sequence is sufficiently long to ensure that the sequence is unique. Such considerations are well known and are substantially the same considerations used in selecting 15 a probe for various techniques, such as for *in situ* hybridization (ISH).

When distances between the anchor sequence and probe are to be determined, the anchor sequence is sufficiently long and sufficiently complementary to the 20 target sequence in the sample oligonucleotide sequence to ensure that the sample oligonucleotide sequence remains hybridized to the anchor sequence in the presence of the field used to extend the sample oligonucleotide sequence. Preferably the anchor 25 sequence is from about 10 to about 100 bases, preferably from about 20 to about 40 bases, most preferably about 30 bases.

As the length of the anchor sequence increases, the degree of specificity for a unique sequence 30 increases. In addition, the strength of the bonds formed by hybridization increases as the length of the anchor sequence increases, providing the sample oligonucleotide sequence/anchor sequence complex with the ability to remain a complex and thus immobilized in 35 a stronger field. However, longer oligonucleotide sequences are more difficult and expensive to prepare

than shorter oligonucleotide sequences. Therefore, these conflicting interests are balanced in selecting the length of the anchor sequence.

As in ISH applications, the anchor sequence need  
5 not be the Watson Crick complementary sequence to the target sequence on the sample oligonucleotide sequence so long as the binding between the anchor sequence and the sample oligonucleotide sequence is sufficient to immobilize the sample oligonucleotide sequence, as  
10 stated above. However, the anchor sequence is preferably complementary to the target oligonucleotide sequence in the sample oligonucleotide sequence to optimize the ability of the sample oligonucleotide sequence/anchor sequence complex to remain complexed  
15 when subjected to the field.

Methods for selecting and synthesizing such oligonucleotide sequences are well known. In addition, considerations for selecting anchor sequences for particular applications are described in detail  
20 hereinafter.

The anchor sequence can be bound to the support either directly or through a linking group by any of the methods described hereinafter. The linking group can be an irrelevant oligonucleotide sequence which  
25 allows the anchor sequence to extend from the surface of the support to facilitate binding of the entire anchor sequence to a sample oligonucleotide sequence. Such irrelevant oligonucleotide sequences are preferably sequences which are flexible and can bend,  
30 such as for example, poly-T having at least three nucleotides, preferably at least 5 nucleotides.

Alternatively, the linking group can be a polypeptide, preferably a relatively short polypeptide. Methods for linking peptides or proteins to nucleic  
35 acids and to the solid phase are discussed above. The peptide linker is preferably from four to twenty,

preferably from five to 10, amino acid residues in length. The peptide does not include with a large number of positively charged groups to avoid non-specific binding of oligonucleotide. The peptide 5 preferably also does not have a large number of negative charges to facilitate binding to the anchor sequence. Preferably the peptide does not include very hydrophobic amino acids, such as leucine, because leucine is relatively insoluble, thus difficult to work 10 with, and might bind to the surface of the polystyrene bead. A polypeptide with one amino group at one end and one carboxyl group at the other is preferred. Homopolymers are preferred.

Most preferred linkers are polyglycine, 15 polyalanine, and polyproline. Polyalanine forms an alpha helix which would hold the anchor sequence away from the support surface or, when linking the probe to the label, would hold the probe oligonucleotide sequence out from the bead. Polyproline forms a rigid 20 coil (not an alpha helix), which can also be used to hold the oligonucleotide sequence out from bead or support. In addition, a large variety of other small organic molecules can also be used.

The anchor can be linked to the solid support at 25 an internal oligonucleotide in the anchor sequence, so that there are two "arms" of the anchor sequence hanging from the linker. A restriction site near the end of one of the arms then provides a means for cutting that arm off. DNA with a linker site anywhere 30 in the sequence is available commercially from; e.g., The Midland Certified Reagent Company (Midland, Texas) and Peninsula Laboratories (Belmont, California).

The support to which the anchor sequence is immobilized can be any solid phase on which 35 hybridization can be performed. The solid phase is preferably a glass or plastic surface, conveniently a

microscope slide. For use with an epifluorescent microscope, the slide is preferably opaque to absorb light which may otherwise be reflected. A preferred support is described hereinafter in describing a  
5 preferred device of this invention.

The anchor sequence is bound to the support by conventional methods. The goal of the surface chemistry is to attach a few sample oligonucleotide sequence DNA to the support through hybridization with  
10 the anchor sequence, as it is necessary to observe only a few molecules. Thus, the chemistry need not be very efficient. This is in contrast to the requirements in, for example, procedures for solid support DNA synthesis machines, where the goal is to produce as much product  
15 as possible. Therefore, a number of techniques are suitable.

For example, to attach the anchor sequence to the support, a portion of the support is coated with a solution of gelatin, which is then allowed to dry into  
20 a film coating the surface of the support. The gelatin is then crosslinked to make it irreversibly insoluble using standard techniques of histological specimen preparation. Such techniques are well known and are described in Galigher and Kozloff, *Essentials of  
25 Practical Microtechnique*, Lea & Febiger, Philadelphia, (1964) for example. Activated anchor oligonucleotides are then covalently attached at the free amino groups of the gelatin.

Alternatively, methods used for synthesis of DNA  
30 involve a solid, silica-based (e.g., glass) support, having the 3' nucleotide covalently linked to the support. This linkage is accomplished by reacting the nucleotide 3'-p-nitrophenylsuccinate ester with a free amino group on the surface of the support. (See  
35 Caruthers, *Science* 230:281-285 (1985) for a review of automated oligonucleotide synthesis methods.) The

method can also be used to attach the anchor sequence to a glass or other silica-based support.

For RNA anchor sequences, the anchor sequence can also be linked by either the 2' or 3' hydroxyl on 5 ribose by well known chemistry.

Alternatively, a metal, such as gold, silver, or copper, can be attached to the support and used to bind the anchor sequence to the support. Conveniently, the metal can also function as the electrode. There are 10 numerous articles on the reaction of thiols with gold, silver, and copper surfaces which can be used to attach the anchor sequence to the metal and thus to the support. (See for example, Laibinis et al., *J. Am. Chem. Soc.* 114:1990-1995 (1992).)

15 In addition, Whitesell et al., *Science* 261:73-76 (1993) describe the preparation of a gold surface which has been deposited by evaporation onto glass. The gold surface is coated with an aminotrithiol "tripod". The three thiol groups bind tightly to the gold surface, 20 and the amino groups are exposed at the opposite, free surface. Although the authors describe linking polypeptides to the free amino groups, nucleotides could also be attached, using the chemistry described previously. Bifunctional organic disulfides also bind 25 tightly to gold surfaces. (See for example Nuzzo et al., *J. Am. Chem. Soc.* 105:4481-4483 (1983).)

Using a metal surface is advantageous because the metal surface can also be used as an electrode to produce an electric field. Gold is preferred because 30 it is generally chemically inert. In addition, fabrication of micro patterns of gold films, used in the computer chip industry, is well known and is commercially available. However, with many electrolytes, gas is evolved at the electrode surface, 35 and may interfere with observation. Thus, it may be advantageous to use an alternating high frequency

electric field to extend the DNA (see Kabata et al., *Science* 262:1561-1563 (1993)), since no gas is evolved when there is no net current.

Photoresist fabrication techniques can also be  
5 used to produce patterns of methylsilane and  
aminosilane on the surface of glass. (See Britland  
et al., *Biotech Prog.* 8:155-160 (1992).) The  
methylsilane produces a non-reactive surface, while  
peptides and proteins (and nucleotides) can be coupled  
10 to the aminosilane surface using chemistry described  
above when the anchor sequence is linked to a peptide.

#### The Probe

A probe of this invention is an oligonucleotide  
15 sequence which hybridizes to a target region of the  
sample oligonucleotide sequence. As with the anchor  
sequence, the probe can be a DNA sequence or an RNA  
sequence is preferably single-stranded to maximize the  
strength of the bond between the probe and the sample  
20 oligonucleotide sequence.

As with the anchor sequence, the probe can  
hybridize with the target sequence in the sample  
oligonucleotide sequence by Watson Crick or triple  
helix binding. However, use of a single stranded probe  
25 and sample oligonucleotide sequence was preferred. The  
same general considerations regarding the percentage  
and location of complementary oligonucleotides are  
involved for the probe as for the anchor sequence.  
However, when the sample oligonucleotide sequence is  
30 extended and the probe is separated from the  
hybridization mixture by an electrical field, the probe  
need not be as tightly bound to the sample  
oligonucleotide sequence as the anchor sequence. The  
anchor sequence experiences the force of the electric  
35 field acting on the entire length of the sample  
sequence. The probe only experiences the force on the

length of the probe. Therefore, a relatively short probe can be used and remain bound (hybridized) in a field which requires a substantially longer anchor sequence for the anchor sequence to remain hybridized.

5

If the probe is attached to a magnetic particle, the considerations would be the same as for an anchor sequence which hybridizes to a sample oligonucleotide sequence which is bound to the magnetic particle. That 10 is, the probe would need to be the same length as an anchor sequence attached to a particle of the same magnetic field strength. However, to use shorter probes, a particle having a lower magnetic field strength can be used. Thus, the same size probes can 15 be used whether the separation means is a magnetic or electric field.

The length of the probe on the target region of the sample oligonucleotide sequence to which the probe binds depends on the analysis to be performed. In 20 general, the criteria for selection of probe length and target sequence do not differ from those of the prior art, with the exceptions described above for use of magnetic particle-labeled probes. For example, general considerations for selection of appropriate probes are 25 usually those used for selection of probes for fluorescent in situ hybridization (FISH) methods. FISH is a technique used to detect, quantitate and localize RNA and DNA in cells and tissues, usually in fresh frozen or paraffin-embedded tissue sections. In FISH 30 methods, a fluorochrome-labeled probe oligonucleotide sequence is incubated with the tissue section to hybridize the probe to a sample oligonucleotide sequence.

When one wants to determine whether two 35 oligonucleotide sequences are on the same molecule of DNA, such as for mapping applications, the anchor

sequence is selected to bind to the first oligonucleotide sequence and the probe is selected to bind to the second oligonucleotide sequence.

Considerations for selection of oligonucleotide

- 5 sequence for such probes does not differ from those for probes for in situ hybridization techniques and are well known. The probes can be any sequence which hybridizes specifically to the second oligonucleotide sequence under the conditions for the method. The  
10 probes are preferably from about 10 to about 100 bases, preferably from about 20 to about 40 bases, most preferably about 30 bases.

For preparing characteristic patterns for sample DNA for identity typing applications, such as in forensics or paternity determinations, the present invention uses relatively short oligonucleotide sequences, on the order of about 5 to 15, usually 7 to 10, nucleotides of arbitrary sequence. Such oligonucleotide sequences can be selected to bind  
20 approximately every 1,000 (for probes of 5 bases) to 1,000,000,000 (for probes of 15 bases) bases of genomic DNA, depending on the length of the probe sequence. An arbitrary nucleotide sequence of 6 bases binds once approximately every 4,000 bases, and a probe of 10  
25 nucleotides binds once approximately every 1,000,000 nucleotides. Most preferred are probes of 7 or 8 nucleotides of arbitrary sequence.

However, if binding every 4,000 nucleotides is desired and a probe of six nucleotides is not  
30 sufficiently long to remain hybridized during the analysis, the probe can be effectively made ten nucleotides in length while retaining the same specificity by synthesizing a plurality of probes. The probes each have the selected six nucleotides. The  
35 probe is elongated by making four versions of the probe at each of the remaining four positions to produce a

probe ten nucleotides in length. That is, 256 probes of different specificity, each with the same six nucleotides and every combination of nucleotides at the remaining four positions can be synthesized. Of course, the concentration of the probes would have to be increase 256-fold in this example to maintain the same molarity of the specific probe for efficient hybridization. See page 6.9 in Ausubel, Short Protocols in Molecular Biology, Second Edition, John Wiley and Sons, New York (1992). In this way, the desired frequency of binding can be achieved while maintaining a sufficient strength of binding to remain hybridized during the analysis.

Selection of such oligonucleotide sequences for use as primers is well known and is described in International Application No. PCT/US91/00841 (Publication No. WO 91/14001, published September 19, 1991) to Livak et al., (the RAPD method), for example.

When the probe is to hybridize with a target sequence of interest, such as an oligonucleotide sequence characteristic of a disease gene, the probe is generally at least about 15, preferably at least about 20 nucleotides to ensure the desired specificity. The probe sequence is preferably 10 to about 100 bases, preferably from about 20 to about 40 bases, most preferably about 30 bases. Probes of about 20 to about 30 nucleotides can be selected to be specific to a target oligonucleotide sequence and are readily synthesized.

In addition, the probes are labeled with a fluorochrome. The fluorochrome provides essentially a point source of light which facilitates detection of the probes on the sample oligonucleotide sequence. Fluorochromes are well known and include fluorescein (e.g., fluorescein isothiocyanate - FITC), rhodamine (e.g., tetramethylrhodamine isothiocyanate - TRITC),

phycoerythrin (PE), allophycocyanin (APC) and Texas Red (Molecular Probes, Eugene, Oregon).

A most preferred fluorescent label is a fluorochrome incorporated into a plastic bead, 5 preferably a polystyrene bead of controlled diameter. The fluorochrome is incorporated into a plastic bead during the manufacture of the beads to avoid having the dye molecules to leach away from the bead during the incorporation process. The fluorochromes are protected 10 from the photobleaching that plagues conventional FISH methods, since oxygen and oxygen radicals cannot readily reach the dye molecules. The spheres can contain many molecules of fluorochrome, providing a bright image which is readily detectable and is 15 particularly advantageous for image analysis techniques.

Fluorochrome-containing polystyrene spheres can be purchased from Duke Scientific Corporation (Palo Alto, California). Green, red, and blue 20 fluorochrome-containing spheres 0.5 microns in diameter are available with reagents that covalently link peptides to the plastic. In particular, beads that can be linked to carboxyl groups on peptides are commercially available under the tradename FX 25 COVASPHERES from Duke Scientific Corporation. Beads of other, smaller and larger diameters are also commercially available.

Techniques for binding a fluorochrome to an oligonucleotide sequence are well known and are 30 utilized in fluorescent in situ hybridization techniques. As stated previously, in FISH methods, a fluorochrome-labeled probe oligonucleotide sequence is incubated with the tissue section to hybridize the probe to a sample oligonucleotide sequence.

35 The probes are typically labeled with a fluorochrome using one of three techniques. In a first

technique, the probe is conjugated to the fluorochrome. See, for example, Bauman et al., in *Flow Cytogenetics*, J. W. Grey Editor, Academic Press, London, 1989, and which describes methods for binding fluorochromes to 5 oligonucleotide sequences.

In a second method, the probe has a biotin covalently attached to one or more nucleotides. The sample oligonucleotide sequence is contacted with avidin which has been labeled with a fluorochrome. 10 Avidin binds tightly to biotin, and thus the probe is labeled. The hybridization of probe and sample can be performed simultaneously with contacting the probe with avidin. Alternatively, the hybridization of probe and sample can be performed as a first step, and incubation 15 with the avidin is done following hybridization. The separation of steps is particularly useful when the hybridization conditions are sufficiently harsh that the avidin could be denatured and inactivated.

In the third technique, the probe has digoxigenin 20 covalently attached to one or more nucleotides, and a fluorochrome-labeled anti-digoxigenin antibody binds the digoxigenin. This procedure is analogous to the biotin/avidin procedure described above. Digoxigenin is used because extremely high affinity 25 anti-digoxigenin antibodies are commercially available.

FISH techniques are within the level of skill in the art and are reviewed in Leversha, *Med. J. Australia* 158:545-551 (1993). Other articles describing FISH techniques include Trask, *TIG* 7:149-154 30 (1991); Brandriff et al., *Envirn. Mol. Mutagen* 18:259-262 (1991); Bauman et al., In: Gray JW, eds. *Flow Cytogenetics*, London: Academic Press, 275-301 (1989); Pinkel et al., *Proc. Natl. Acad. Sci. USA* 83:2934-2938 (1986); and Pinkel et al., *Proc. Natl. 35 Acad. Sci. USA* 85:9138-9142 (1988). Specific laboratory directions for *in situ* hybridization,

exemplified by detection of cellular RNA using radioactive probes, are given on pages 14-2 to 14-19 of Ausubel, Short Protocols in Molecular Biology, Second Edition, John Wiley and Sons, New York (1992).

5        In a preferred labeled probe construct, one end of a polypeptide spacer is bound to the probe, and the other end of the spacer is bound to a fluorescent bead. Preferably, the amino terminus of a polypeptide spacer is covalently bound to the probe, and the carboxy 10 terminus is covalently bound to the fluorescent bead. The peptide spacer can be bound to any portion of the probe. However, when the bead has a large negative charge, the bead is preferably attached at the end of the probe distal to the anchor, so that the force of 15 the electric field is distributed along the length of the probe, rather than experienced by the nucleotides at the proximal end of the probe, and thus removed from the sample DNA.

The spacers can be the same as the anchor sequence 20 linking sequences described above. A preferred polypeptide spacer is polyglycine, having from four to twenty, preferably from five to 10 glycines. Oligonucleotides that can be coupled to free amino groups are commercially available. Such 25 oligonucleotides are readily coupled to the amino group of a linker peptide which has been coupled to the bead.

In applications where a plurality of probes are used to bind to a plurality of target regions in the sample oligonucleotide sequence, each probe can be 30 labeled with a different fluorochrome. Combinations of fluorochromes used for labeling are chosen so that distinguishable wavelengths of light are emitted. For example, use of three different fluorochromes of 35 distinguishable colors facilitates the identification of seven different probes. (See Ried et al., Proc. Natl. Acad. Sci. USA 89:1388-1392 (1992) which

describes the identification of seven different probes using three fluorochromes in a fluorescent in situ hybridization method.)

By controlling the intensity of the light emitted by each of the fluorescent plastic beads, different intensity levels can also be measured, distinguishing probes with one bead from those with two or more beads. Together with use of different colors, large numbers of different probes can be distinguished. The intensity of light emitted at different wavelengths by different types of beads is determined by the total amount of the relevant fluorochrome in the bead.

#### The Device

A DNA analysis device 100 of this invention has a generally planar support 110 having a first end 111 and a second end 112 which is opposite to, removed from, and substantially parallel to first end 111. Support 110 can be glass or plastic, and is conveniently a microscope slide. Support 110 can be transparent or opaque. The support is preferably opaque when used with an epifluorescent microscope.

A first electrode 120 is preferably fixedly attached to a planar surface 113 of support 110 adjacent to but separated from first end 111 of support 110. A second electrode 130 is preferably fixedly attached to planar surface 113 of support 110 adjacent to but separated from second end 112 of support 110. The spacings of first and second electrodes 120, 130 from first and second ends 111, 112, respectively, is not critical. The important aspect as explained more completely below is the spacing between electrodes 120, 130.

Electrodes 120, 130 are made of an electrically conductive material, preferably copper, silver, or more preferably, gold. In one embodiment, electrodes 120,

130 are small rectangles, on the order of 5 X 5 mm which are formed from thin, flat sheets, e.g., a foil, of the selected metal. First and second electrodes 120, 130 have approximately the same thickness. The  
5 anchor sequence is immobilized on support 110 adjacent first electrode 120, and, preferably, on first electrode 120. The anchor sequence is immobilized on first electrode 120 in region 140, i.e., a middle region of electrode 120, and extends toward a portion 113A of  
10 surface 113.

To attach the anchor sequence to first electrode 120, at least a portion 140 of electrode 120 which is exposed to an interior of device 100, conveniently the entire electrode, is coated with a solution of gelatin,  
15 which is then allowed to dry into a film coating the surface of electrode 120. The gelatin is then crosslinked and attached to the anchor sequence as described previously. Alternatively, electrode 120 can be coated with an aminothiol and the anchor  
20 oligonucleotides are then attached as described above. Following attachment of the anchor sequence or a linking group, first electrode 120 is attached to surface 113 of support 110 by glue such as an epoxy adhesive. Second electrode 130 is also attached to  
25 surface 113 by glue.

A substantially planar cover (not shown in Figure 1 for clarity) extends at least from first electrode 120 to second electrode 130. The cover is preferably transparent for most uses but can be opaque when used  
30 with an inverted epifluorescent microscope. In one embodiment, the cover rests on electrodes 120, 130. In another embodiment, the cover is attached to electrodes 120, 130 by glue. The cover can be glass or plastic or any other transparent material. Conveniently, the  
35 cover is a conventional microscope coverslip.

Placing the cover on electrodes 120, 130 forms a

detection chamber bounded by edges 120A and 130A of electrodes 120 and 130, respectively; a portion 113A of surface 113 between electrodes 123; and the surface of the cover between electrodes 120 and 130. The  
5 detection chamber has a size of h x a x b, where a and b are preferably on the order of 10 mm each and h is preferably on the order of one millimeter. One of the remaining sides of the detection chamber can be sealed with a material which does not interfere with the  
10 hybridization process, preferably small strips of glass or plastic which can be positioned after addition of the hybridization reagents. Alternatively, the detection chamber can be sealed on one or both of the remaining sides with rubber cement. Device 100 is  
15 preferably supplied with the anchor oligonucleotides attached, preferably in a desiccated container to avoid possible degradation of the anchor oligonucleotide.

First and second electrodes 120, 130 are attached to wires 150 and 160, respectively, for electrical  
20 connection to a power supply. Preferably, wires 150 and 160 are each a 28 gauge wire. (28 gauge wire has a 0.65 mm diameter.) Each 28 gauge wire is connected to a separate terminal. Larger wires, which in turn connect to the power supply, are also connected to the  
25 terminals.

When a first voltage level is applied to first electrode 120 and a second voltage level, different from the first voltage level, is applied to second electrode 130, an electric field is formed between  
30 electrodes 120 and 130 which acts on the anchor sequence as described above.

In another embodiment, as illustrated in Figure 2, first electrode 120 (Fig. 1) is replaced with a composite electrode 220 having three sandwiched  
35 electrode layers 220-1, 220-2, and 220-3 that are formed from three sheets of metal. The sheets of metal

are conveniently of equal thickness. The thickness of the metal foil is selected so that the thickness of composite electrode 220 is approximately equal to the thickness of second electrode 230.

5        Prior to assembling composite electrode 220, the anchor sequence, or a linking group which facilitates subsequent attachment of the anchor sequence is bonded to central electrode layer 220-2 in the same manner as described above for first electrode 120 and that  
10      description is incorporated herein by reference. After central electrode layer 220-2 is coated, layer 220-2 is sandwiched between electrode layers 220-1 and 220-3 so that electrode layer 220-2 is in the center of composite electrode 220.

15      First electrode 220 and second electrode 230 are attached adjacent to, but removed from opposite ends of surface 213 of support 210 in a manner similar to that described above for electrodes 120 and 130 of Figure 1. After attachment to surface 213, first electrode 220  
20      and second electrode 230 are covered by cover 270 to form detection chamber 250. The anchor sequence is only exposed to detection chamber 250 at edge surface 221 of center electrode layer 220-2. Two outside electrode layers 220-1 and 220-2 of composite  
25      electrode 220 not only make electrode 220 the same height as second electrode 230 but also restrict the sample oligonucleotide sequence to a central zone of detection chamber 250, while ensuring a uniform, parallel electric field to extend the sample  
30      oligonucleotide sequence. Sample oligonucleotide sequence 280 is shown after hybridization of the sample to the anchor sequence.

The depth of field (focus) of a microscope at high power is sufficiently narrow so that one could just  
35      focus on a middle zone of composite electrode 220. Any distorted oligonucleotide sequence molecules at the

electrode edges would be sufficiently out of focus that so that no interference with observation of molecules with the desired orientation would occur.

Figures 3 and 4 illustrate another embodiment of a 5 DNA analysis device 300 of this invention. Preparation and use of one embodiment of device 300 is described in detail in Example 4. In Figure 3, support 310, conveniently a large coverslip, has a generally planar surface 313, with a first end 311 and a second end 312 which is opposite to and removed from first end 311. 10 Support 310 is prepared as described in Example 4.

First electrode 320 is placed directly on surface 313 adjacent to but separated from first end 311.

Second electrode 330 is placed on surface 313 adjacent 15 to but separated from second end 312 of surface 310. As in the previous embodiments described above, the spacing of electrodes 320 and 330 from ends 311 and 312, respectively, is not critical. First electrode 320 and second electrode 330 are round wire electrodes 20 for electrical connection to a power supply. First and second electrodes 320 and 330 are approximately the same size wire.

In the embodiment described in Example 4, DNA 25 analysis device 300 is mounted on a transparent stable dielectric plastic sheet. The plastic sheet is not shown in Figures 3 and 4, because the plastic sheet is not an important feature of the invention. Moreover, dimensions given herein are illustrative only and are not intended to limit the invention to the particular 30 dimensions given. In view of this disclosure, those skilled in the art can implement DNA analysis device 300 in a wide variety of sizes, shapes, and forms.

In the embodiment of Example 4, two brass bolts 35 are passed through two holes, approximately 50 mm apart, in the plastic sheet and nuts attached to the bolts. Electrode 320 is wound around one of the bolts

and the nut is tightened against electrode 320. Electrode 320 is wound around the other of the bolts and the nut is tightened against electrode 330. Thus, electrodes 320 and 330 are spaced approximately 50 mm apart. Electrodes 320 and 330, after attachment to the bolts, have some springiness and so support 310 is slid under electrodes 320 and 330, and is held in place by electrodes 320 and 330.

A cavity is formed through a gel to form a gel frame 350 so that gel frame 350 has an exterior surface 350A and an interior surface 350B. Gel frame 350 is made of a material suitable for electrophoresis of nucleic acid, and is preferably made from a polyacrylamide gel sheet of uniform thickness.

Although illustrated as a square, the cavity can be of any shape. Generally, the shape of exterior surface 350A is the same as the shape of interior surface 350B. In the embodiment of Example 4, exterior surface 350A is the exterior surface of a 10 x 10 mm square and interior surface 350B is the surface of a 5 x 5 mm square. The vertical thickness of gel frame 350 is one millimeter.

Gel frame 350 with the cavity is placed on surface 313 so that a center of the cavity is approximately centered between electrodes 320 and 330, and so that the cavity forms an electrophoresis chamber 340, hereinafter chamber 340, having a bottom surface that is a portion of surface 313 and a vertical surface that is inner surface 350B of gel frame 350. Chamber 340 is filled with a buffer solution (an electrophoresis buffer) containing an anchor oligonucleotide sequence affixed to a solid support, conveniently an agarose ball as described in Example 4 (not shown).

Pools 360 and 380 are drops of a solution that conducts electricity from electrodes 320 and 330,

respectively, to gel frame 350. Conveniently, pools 360 and 380 are drops of the electrophoresis buffer used for the DNA analysis in chamber 340.

A cover 370, conveniently a small coverslip, is placed on top of gel frame 350, following loading of an anchor oligonucleotide sequence into chamber 340, to seal chamber 340 by forming a top surface that is opposite and removed from the bottom surface that is a portion of surface 313. Cover 370 can be any solid, material that covers, and thereby seals, chamber 340. Cover 370 is conveniently made of a transparent material to facilitate observation of materials in chamber 340.

Figure 4 is a side view of DNA analysis device 300 of this invention. First electrode 320 and second electrode 330 are shown in pools 360 and 380, respectively that are formed on planar surface 313 of support 310. Gel frame 350 is covered by cover 370.

20                   The Method

The method is described generally below, followed by various embodiments of the method adapted for particular analysis techniques.

The first step in performing the present method is contacting a sample oligonucleotide sequence with an anchor sequence of this invention and with a probe in a suitable buffer to form a complex between the sample oligonucleotide sequence and the anchor sequence. The sample oligonucleotide sequence and probe can be mixed prior to contact with the anchor sequence, or the sample oligonucleotide sequence can be contacted with the anchor sequence and then the probe can be added to form a hybridization mixture. Following hybridization, unbound probe and, preferably, unbound sample oligonucleotide sequence are removed. Any probe which binds to a sample oligonucleotide sequence which is

immobilized to the support through the hybridization to the anchor sequence is then detected.

The conditions for hybridization of oligonucleotide sequences are well known. Generally, 5 the hybridization step is either performed in a buffered aqueous salt solution at high temperature or in the presence of formamide at lower temperature. The aqueous, high temperature procedure is typically carried out in a Tris buffer, such as 0.3 M NaCl, 20 mM 10 Tris-HCl, pH 6.8, at 67°C. Other buffering systems such as hepes or glycine-NaOH and potassium phosphate buffers can be used.

One to ten nanograms of the probe is generally used per hybridization mixture. However, when using 15 probes with a lower sensitivity, increased probe concentrations; e.g., 250-750 ng/ml in the hybridization solution. Such high probe concentrations facilitates short hybridization times of about one to two hours.

20 When the probe is attached to a bead, the size of the bead used is a consideration at high probe concentrations. For example, if the bead is 1 micron in diameter, a concentration of 2 nM would be almost solid beads. 2 nM would be 7 ng/ml for a 10 base 25 probe. If the beads were only 0.1 microns in diameter, a concentration 1000 times higher can be used. Preferably, the beads are from about 0.1 to about 0.2 microns in diameter. Such sizes not only allow use of 30 probes at higher concentrations, but also facilitate faster hybridization, because the diffusion coefficients are higher.

Typically, the total volume of the buffer containing the sample oligonucleotide sequence and probes (the hybridization mixture) required is about 15 35 to 20 µl. For use in a device of this invention, rather used as a drop placed on a slide or other

support, the dimensions of the device determine the required volume. If the device is 1 mm<sup>3</sup>, the required volume is one  $\mu$ l. Similarly, if the device is 2 X 5 X 5 mm, the volume would be 50  $\mu$ l. A preferred device of 5 this invention having a detection chamber of 1 X 10 X 10 mm requires a volume of 100  $\mu$ l.

When added to the assay device, the hybridization mixture is covered, as with a coverslip or by placing in a sealed device to prevent evaporation of the 10 reagents. Generally, the hybridization step is performed 12 to 14 hours at 37-42°C in a chamber humidified with the hybridization buffer.

Alternatively, the hybridization is performed in formamide at lower temperatures. The formamide 15 procedure is generally used with thermally sensitive probes. For example, biotinylated probes are less thermally stable than radioactive probes. With such probes, the formamide concentration is generally lowered from about 50 to about 45% in otherwise 20 standard *in situ* hybridization conditions. Typically, the hybridization is carried out in about 40% to 50% formamide in SSC (0.15 M NaCl, 0.015 trisodium citrate, pH 7.0 (adjusted with NaOH). The probe and carrier DNA or RNA concentrations are the same as for the aqueous 25 buffer solution. A preferred formamide hybridization solution is described in the Examples.

Because the anchor sequences are immobilized in a selected region on the support, the rate of hybridization of sample oligonucleotide sequence 30 molecules to anchor sequence molecules is much slower than would occur if the two species were in solution. The rate can be increased by using the electric field to concentrate the sample molecules against the anchor surface. In this embodiment, the field is applied in 35 the direction opposite to that used to extend the sample oligonucleotide sequence molecules after

hybridized to the anchor sequence molecules. In particular, for concentration of the probes, the anchor sequence region is positive while for extension the anchor sequence region is negative. Such concentration 5 increases the speed and thus the efficiency of hybridization by several orders of magnitude, and allow analysis of a small number of sample oligonucleotide sequence molecules. Typically, a field of 10 to 100 V/cm would be applied for 10 minutes to achieve 10 concentration, then would be turned off for 1 min to allow free diffusion on the support. These cycles are preferably repeated for the duration of the hybridization.

After the sample molecules are hybridized to the 15 anchor molecules, they are also highly concentrated against the surface. Thus hybridization of probe molecules to sample would also be accelerated by periodic application of the field as described above.

The anchor sequence/sample oligonucleotide 20 sequence/probe complex is subjected to a separation means which moves unbound oligonucleotide sequences away from the anchor sequence. In a preferred embodiment, the separation means is an electric field which moves unbound probe and any unbound sample 25 oligonucleotide sequences away from the anchor sequence in the direction of the field based on the charges inherent in oligonucleotide sequences.

The force of the electric field is sufficient to move unbound probe and any unbound sample 30 oligonucleotide sequences in the direction of the field based on the charge on oligonucleotide sequences. However, the force is not sufficient to break long 35 sample oligonucleotide sequences or to break the bonds in sample oligonucleotide sequence/anchor sequence complexes and thus remove sample oligonucleotide sequences which hybridized to the anchor sequence.

In general, the field should be about 1 to about 1000 V/cm, preferably 10 to 100 V/cm. A lower voltage is used when the sample oligonucleotide sequence is longer to avoid breaking the oligonucleotide sequence.

- 5 In general the maximum voltage is inversely proportional to the length of the sample oligonucleotide. The time for extension of the sample oligonucleotide sequence is less than one minute. Time for removal of the unbound probes is 1 to 10 minutes.
- 10 Therefore, the probes are preferably not detected until after 10 minutes in the electric field.

In any analysis where the position of the probes in relation to the anchor sequence is determined, the sample DNA must be extended. When the analysis is based on binding of a probe to the sample oligonucleotide sequence, as in determining whether two oligonucleotide sequences are on the same molecule of DNA or when presence of a disease gene sequence is detected, the sample oligonucleotide sequence need not be extended. However, even in such analyses, the sample oligonucleotide sequence is preferably extended to determine the distance of the probe from the anchor. This distance additionally provides information regarding the distance between two sequences of unknown location. Alternatively, when detecting a target oligonucleotide sequence of a disease gene, the distance can be used to confirm that the probe sequence is bound in the proper location in relation to the anchor sequence.

30 Extension of the sample oligonucleotide sequence can be performed using a magnetic bead at an end of the portion of the sample oligonucleotide sequence to be analyzed. Alternatively, the magnetic bead can be placed on the probes when the probes are sufficiently long to remain hybridized in the magnetic field.

Whatever the purpose of the assay, unbound probes

must be removed.. This can be done by extensive washing. Preferably, the probe can be attached to a magnetic bead or removed by use of an electric field which moves the charged probes in the direction of the  
5 field.

Alternatively, the separation means can be a magnetic field. In that embodiment, magnetic particles are bound to either the sample oligonucleotide sequence or, preferably, the probe prior to hybridization. Like  
10 the electric field, the magnetic field moves magnetic particle-labeled probe away from the anchor sequence in the direction of the field. In addition, probes which hybridize to the sample oligonucleotide sequence effectively provide a magnetic particle-labeled sample  
15 oligonucleotide sequence. If the probes are not attached to a magnetic particle, the probes can be removed by washing or by use of an electric field.

Thereafter, whether the probe is bound to the sample oligonucleotide sequence is determined by  
20 observing the presence of fluorescent-labeled probes on the support while the field is on. In addition, the positions of the bound probes, in relation to the anchor sequence can also be determined. The presence or location of the fluorescent probes can be determined  
25 with a fluorescent microscope, preferably an epifluorescent microscope, such as those commercially available for analysis of FISH assays. As is well known, the better the optics and the mechanical stability of the microscope, the higher the resolution  
30 which can be achieved.

The positions of the images of the probes in a field of view could be determined manually by comparison with a reticule in the eyepiece. When the sample oligonucleotide sequence is single-stranded DNA  
35 or RNA, the sample oligonucleotide sequence extends approximately 0.6 nm for each base (0.6 microns for

each kilobase or 0.6 mm for each megabase).

A preferred detection technique for analysis of complicated probe patterns (e.g., as can be produced in some mapping embodiments or when the positions of

5 several different probes are to be determined) is use of an electronic image detector (e.g., a TV camera), and a computer to analyze the image from the microscope and to control an X-Y stage holding the sample.

Computer-implemented image analysis decreases the  
10 manual work required and can facilitate more accurate measurements of the positions of the probes.

To determine the position of a probe from the anchor as accurately as possible, a fluorescent bead attached to the probe should be no larger, and is  
15 preferably smaller than the wavelength of light emitted, which is typically 500 nm. If, for example, a bead 100 nm in diameter is used, the diameter of the image of the bead is larger by an amount approximately equal to the wavelength of light (i.e.; about 600 nm in  
20 diameter). The edge of the image of the bead appears diffuse due to the wave nature of light and distortions and imperfections in the optics of the microscope.

The position of the center of the disc-shaped image can be determined accurately by measuring the  
25 intensity of the image at many points, and thereby determining the center. One of many algorithms for determining the center would be to compute the center of intensity which is analogous to the center of mass. Many such algorithms are known and published.

30 To accurately determine the position of a bead, the diameter of the image of the bead should occupy at least about 1/100th of the linear field of view. Thus, if the image is detected using a conventional charge coupled device (CCD) camera, having about 1000 X 1000  
35 pixels in a square array, the image is about 10 pixels across. The image then occupies about 80 pixels (a

circle 10 pixels in diameter), and thus 80 values of its intensity are obtained. Such a field of view is 60 microns (100 X 600 nm), and thus encompasses about 100 kilobases. To characterize a longer sample  
5 oligonucleotide sequence, analysis of many overlapping fields of view, and reconstruction of the image of the entire specimen molecule can be performed. An accurate X-Y stage to move from one to another location to build up the image of the entire specimen molecule can be  
10 used. Such stages are commercially available.

The probes move during analysis due to thermal motion. The distance traveled by the probes, and thus the degree the image occupies a greater space than that of a stationary probe, depends on the time employed to  
15 capture the image. The time required varies depending on the brightness of the image and sensitivity of the camera system. If the system were to determine the probe position at an instant of time, that position would correspond to a particular configuration of the  
20 sample oligonucleotide sequence. However, it is the average configuration of the chain that provides the most useful information, since this average position can be correlated with nucleotide sequence of the sample oligonucleotide. This average position can be  
25 determined by obtaining an image which is blurred over time, since the intensities in that image represent the fraction of the total observation time that the probe was in a given position.

Various ways for performing the method depending  
30 on the type of analysis desired are described below.

#### Mapping

In mapping applications, the present method can determine whether a first oligonucleotide sequence and  
35 a second oligonucleotide sequence are on the same molecule of DNA, and the distance between those

sequences which are on the same molecule. To determine whether a first and a second target oligonucleotide sequence are on a molecule of sample DNA, the sample DNA is contacted with an anchor sequence which

5 hybridizes with the first target oligonucleotide sequence and with a probe which hybridizes with the second target oligonucleotide sequence.

As stated previously, when the sample DNA is not more than about 200 kilobases, no special handling

10 techniques are required. However, since the mapping method is most informative with long DNA sequences, the sample DNA can be greater than about 200 kilobases and special handling is involved. Briefly, the sample DNA is prepared by lysing cells in a gel and adding a

15 fragment of the gel to the support to which the anchor sequence is immobilized.

As in each embodiment of the method, the anchor sequence must be sufficiently long so that it only hybridizes to the first oligonucleotide sequence.

20 Similarly, the probe must hybridize specifically to the second target oligonucleotide sequence. Generally, the probe is from about 10 to about 100 bases, preferably from about 20 to about 40 bases, most preferably about 30 bases.

25 After being subjected to the electric field, the sample oligonucleotide sequence is extended and the distance from the anchor sequence to the labeled probe is determined.

In a preferred embodiment of the method,

30 hybridization of the sample DNA to the anchor sequence forms a double stranded oligonucleotide sequence having a site recognized by a restriction endonuclease and the restriction endonuclease is present in the hybridization buffer or added following hybridization.

35 Following cleavage of the sample oligonucleotide sequence, only the portion of the sample

oligonucleotide sequence which is remains bound to the anchor sequence is immobilized, allowing the determination of both the distance and direction of the probe from the anchor. Whether the 3' or 5' portion of 5 the sequence remains hybridized to the anchor sequence depends on the placement of the restriction site within the anchor sequence. That is, when the restriction site is within a few nucleotides of one end of the anchor sequence, that end of the sample oligonucleotide 10 sequence is does not remain immobilized. In a most preferred embodiment, there are two endonuclease sites, one on each side of the point the anchor nucleotide is attached to the solid support. In this case, either arm of the sample oligonucleotide sequence can be 15 studied, depending on which endonuclease is used.

Prior art fluorescent *in situ* hybridization techniques are capable of determining the position of a unique sequence to within about 10 percent of the length of a chromosome; e.g., about 15 megabase pairs. 20 Mapping the position to higher resolution usually requires construction clones of the DNA and looking for a clone that contains both sequences. If such a clone can be found, the two sequences are then known to be at least as close to each other as the length of the 25 portion of the genome present in the clone.

This typically laborious process is accomplished in several stages, where at the length of the genomic insert in the cloning vector is progressively smaller in order to get higher resolution. If the two 30 sequences are so far apart that even clones with the largest insert can not contain both, intermediate markers must be found, and the sequences related to them. Such progressive searching of a long region of the genome is sometimes called chromosome walking.

35 Direct visual mapping of the location using the method of this invention is clearly less labor

intensive than chromosome walking. To determine the relative location of two oligonucleotide sequences, one of the two sequences must contain a target region which is sufficiently unique so that the region can be used  
5 to hybridize to the anchor sequences. The second oligonucleotide sequence must be sufficiently close to the first oligonucleotide sequence so that both oligonucleotide sequences are on the same molecule of DNA. Alternatively, a third, unique sequence that is  
10 close to (on the same DNA molecule as) the other two must exist.

This method becomes more powerful as the length of the specimen DNA increases, since sites in the genome that are more distant to each other are able to be  
15 related (mapped) to each other without the labor of using intermediate sequences. DNA molecules with lengths in excess of 1 megabases are routinely studied in laboratories using the special techniques discussed previously.

20 As longer sample DNA molecules are examined, the strength of the electric field may need to be decreased in proportion to the length in order to prevent the chain from being pulled apart.

25 There are situations in which one needs to know more sequence information, i.e., the nucleotide sequence of a longer stretch of the DNA, than is obtained by knowing that a short probe sequence has bound to a point on the DNA. One cannot just increase the length of the specific sequence of the probe.  
30 Since the sequence on the specimen DNA is not known, the likely result would just be that the probe would not bind anywhere, and one would only learn that the sequence was not present.

35 If the technique of polymerase chain reaction (PCR) is linked to direct visual mapping, and particularly if several species of distinguishable

probes are used at one time, it is possible to determine a nucleotide sequence toward the end of a chain, use that sequence as a new anchor sequence and thus examine the region beyond it, and so on, i.e., 5 walk the chromosome. The technique is illustrated in the Examples.

Identity Determinations

The method can be used to produce a characteristic 10 pattern for a sample DNA sequence. Such patterns can be used to identify individuals. Preferably, the patterns are also produced in a test DNA sequence for forensic applications or to determine paternity.

The method comprises contacting the sample DNA 15 with an anchor sequence comprising an immobilized, conserved oligonucleotide sequence and with a plurality of probes to form a hybridization mixture. Each of the probes comprises an oligonucleotide sequence of from about 5 to about 15 bases. Considerations for 20 selection of the probe sequences were described previously. In this embodiment, all of the probes can have identical oligonucleotide sequences or a mixture of probes having different oligonucleotide sequences can be used. Following hybridization, the 25 hybridization mixture is subjected to the field to move unbound oligonucleotide sequences away from the anchor sequence and extends the sample DNA in the direction of the field. The positions of the probes in relation to the anchor sequence are determined.

30 Preferably, when the analysis is to determine whether sample DNA and test DNA are from the same individual, the test DNA and the sample DNA are each contacted with the same anchor sequence and probes. Because this technique is based on the actual length of 35 the oligonucleotide sequence between the anchor sequence and the probe (so long as the process of

extending the sample oligonucleotide sequence is completed), the analyses of the sample and test DNA can be run at different times and even under different analysis conditions. The position of the 5 fluorochrome-labeled probes is primarily determined by the structure of the sample molecule. This is in contrast to analysis methods such as polyacrylamide gel electrophoresis in which position is dependent on time of run, voltage, temperature, concentration of gel and 10 so on. Preferably, however, the sample and test DNA are run simultaneously using the same assay conditions.

Following hybridization and separation of unbound oligonucleotide sequences, the positions of the probes from the anchor sequence are determined for the sample 15 DNA and the test DNA. The positions are compared. When the probes are not in the same positions, the sample and test DNA are not from the same individual. When the positions match, the likelihood of a match can be determined statistically by well known methods.

The method can also be used to determine whether a putative father is the father of a child. For such analyses, sample DNA from the putative father, the child and the mother of the child are contacted with the same anchor sequence and probes. Following 20 hybridization and separation of unbound oligonucleotide sequences, the positions of the probes from the anchor sequence are determined for the sample DNA and the test DNA. The positions of the probes on the child's DNA are compared to those of the mother. For any probes in 25 the child's DNA which do not match to those of the mother, the positions of the unmatched probes are compared to the positions of the probes on the putative father's DNA. When those probes are not in the same positions, the putative father is not the father of the 30 child. When the positions match, the likelihood that the putative father is the father of the child can be 35

determined statistically by well known methods.

Detection of Sequences Characteristic of Alleles

In another embodiment, the method is used to  
5 determine whether a test oligonucleotide sequence is  
present in sample oligonucleotide sequence. The method  
comprises contacting the sample DNA with an anchor  
sequence and with a probe comprising an oligonucleotide  
sequence which binds to the test oligonucleotide  
10 sequence. The probe sequence can be any  
oligonucleotide sequence which binds to the test  
oligonucleotide sequence. In one embodiment, the test  
oligonucleotide sequence is sequence which is  
characteristic of a disease gene allele. For example,  
15 the test oligonucleotide sequence can be the F508  
mutation which represents about 70 percent of the  
mutations of the gene for cystic fibrosis transmembrane  
conductance regulator (CFTR), the protein which is  
defective in cystic fibrosis (CF). However, any  
20 oligonucleotide sequence of interest can be detected.  
Other test oligonucleotide sequences can include, for  
example, sequences characteristic of the Y chromosome  
in fetal analyses or an HLA allele for tissue typing.  
In addition, the analysis can be used to detect mRNA  
25 for a particular gene, such as to determine the amount  
of expression of a gene of interest or to determine  
whether an oncogene is being expressed.

As discussed previously, a probe for such analyses  
recognizes a unique region, typically one which  
30 includes a unique sequence characteristic of a gene or  
one or more alleles of a gene. Because the probe is  
used to detect a unique region, the probe is from about  
20 to about 30 bases, as discussed previously.

The method is based on binding of the probe.  
35 However, preferably, the distance of the probe from the  
anchor sequence is determined. This determination can

be used to confirm that the binding is in the correct location and thus, is specific.

This method can be used with a plurality of different probes, each specific for a different test 5 oligonucleotide sequence on the same DNA molecule. The distance from the anchor sequence of any probes which bind can be determined to determine which of the probes bound. Alternatively, each of the probes for a different test oligonucleotide sequence can be labeled 10 with a different fluorochrome and the position of the probes can, preferably, be determined to confirm that the binding is in the correct location and is therefore specific.

This invention is further illustrated by the 15 following specific but non-limiting examples. Procedures which are constructively reduced to practice are described in the present tense, and procedures which have been carried out in the laboratory are set forth in the past tense.

20

#### EXAMPLE 1

##### *Preparation of Reagents and Device*

Preferred reagents and a device of this invention are prepared as described below.

25

##### Oligonucleotide sequences

The oligonucleotide sequences for the anchor and probe are purchased from The Midland Certified Chemical Company, Midland, TX which prepares custom 30 oligonucleotide sequences using phosphoamidite chemistry and purifies them by anion-exchange HPLC. The modified nucleotide containing the amino group can be at either end or at any internal position. They also supply oligonucleotides with an amino group on a 35 6-carbon spacer, which in some cases allows for more efficient binding of the complementary oligonucleotide

sequence.

Assembly of device

Method A. A gold surface (approximately 180 nm thick) on the support is prepared by evaporation on a chromium-coated (approximately 4 nm thick) edge of a 10 X 10 X 1 mm thick silicon slab. The gold surface is then coated by an aminothiol, preferably tris(3-thiolpropyl)glycylaminomethane (HSCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>)<sub>3</sub>CNHCOCH<sub>2</sub>NH<sub>2</sub>, as described in Whitsell and Chang, *Science* 261:73 (1993). Briefly, the gold surface is coated by immersion in a 1 mM deoxygenated ethanolic solution of the aminothiol for 6 hours, as described by Laibinis and Whitesides, *J. Am. Chem. Soc.* 114:1990, (1992). The surface is then washed 3 times with distilled water. In this procedure the thiol groups are bound to the gold while the amino groups remain free on the surface and thus can be linked to other molecules.

Anchor sequence oligonucleotides are coupled to the amino groups on the gold surface by reaction with a primary amino group present on an internal modified nucleotide. The coupling between the two amino groups is accomplished by reaction with glutaraldehyde as described by Alpin and Hughes, *Anal. Biochem.* 113:144 (1981). Briefly, the surface is covered with a 0.25 percent solution of glutaraldehyde in PBS (phosphate buffered saline, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.0) and held at room temperature for 30 minutes. The surface is then washed 3 times in excess PBS.

Two silicon rectangles are attached using epoxy cement to a glass microscope slide which serves as the support with the two gold coated edges parallel and facing each other and about 10 mm apart. One gold surface has been coated with anchor oligonucleotides, the other serves only as an electrode. Two, 4 cm long,

28 gage, nichrome wires are then attached using epoxy cement to the microscope slide, each with one end contacting a gold surface of one electrode, and then leading off the slide to provide electrical connection 5 through longer standard leads to the power supply.

A glass coverslip is placed on top of the two silicon rectangles, creating a 1 X 10 X 10 mm detection chamber, which thus has a volume of 100  $\mu$ l. Fluid is introduced at the edge of the chamber and is drawn in 10 by capillary action. Alternatively, the coverslip is removed, a drop containing 100  $\mu$ l of fluid is applied between the two silicon rectangles, and the coverslip is replaced.

Fluid may be removed, for example in washing 15 procedures, by applying a piece of filter paper at one edge of the chamber and drawing the fluid into the paper. Alternatively, the cover slip may be removed and the entire slide immersed in a small container containing washing solution. This later procedure is 20 used when the temperature must be precisely controlled.

Method B. A gold surface (approximately 180 nm thick) on the support is prepared by evaporation on a chromium-coated (approximately 4 nm thick) edge of a 25 10 X 10 X 1 mm thick glass slab. The gold surface is then removed over a 0.005 X 3 mm rectangle by scratching the gold surface with a sharp needle. Amino groups on anchor sequence oligonucleotides are attached to an amino-derivatized glass surface on the support as 30 described by Alpin and Hughes in *Anal. Biochem.* 113:144 (1981). Briefly, the exposed glass surface is first derivatized by treatment with 3-amino propyl trimethoxy silane (Sigma Chemical Co., St. Louis, MO) for 4 minutes at room temperature. The resulting amino 35 groups on the glass surface of the support and the amino groups on the anchor sequence oligonucleotide are

then cross linked with glutaraldehyde.

Preparation of probes

Polystyrene spheres, approximately 200 nm in  
5 diameter and containing fluorescent dyes (G230 green,  
R200 red) are purchased from Duke Scientific  
Corporation, Palo Alto, California. A pendant  
chloromethyl group is introduced by reaction with HCl  
and formaldehyde, then coupled to Boc-alanine<sub>12</sub> in the  
10 presence of triethylamine, and the protecting group is  
removed from the terminal alanine with trifluoroacetic  
acid in dichloromethane (the standard chemistry used in  
solid phase peptide synthesis). The resulting amino  
group is linked to a 5' amino group on the probe  
15 oligonucleotide using glutaraldehyde. The reaction  
conditions at the addition of Boc-alanine<sub>12</sub> are adjusted  
so that between 10 to 100, preferably 20, peptides are  
added. Thus the beads are finally negatively charged,  
due to the oligonucleotides, with the value of the  
20 charge being about 20 times the number of nucleotides  
in the probe.

Preparation of sample DNA from Blood

DNA is extracted from blood using a QIAGEN blood  
25 DNA kit (Qiagen Inc., Chatsworth, California) according  
to the manufacturer's directions. One ml of whole  
human blood yields approximately 20 micrograms of DNA  
using the Qiagen TIP 20 purification device. After  
purification, DNA is denatured by incubation at 100°C  
30 for 3 minutes, and is diluted immediately into  
hybridization buffer to obtain a final concentration of  
not greater than 1 µg/ml to minimize self-hybridization  
between molecules of sample genomic DNA with  
approximately complementary sequences.

Hybridization

The preferred hybridization procedure is described by Wood et al. in Proc. Natl. Acad. Sci. (US) 82:1585 (1985). The use of tetramethylammonium chloride in the 5 final hybridization buffer results in equal stability for AT and GC base pairs, and thus the dissociation temperature of an oligonucleotide hybrid is dependent only on the length of the complementary sequence and not on its base composition.

10 Briefly, the Wood et al. hybridization procedure consists of five steps (a through e) described below:

a. prehybridization: The chamber and attached anchor sequences are incubated with a solution that coats surfaces which could bind oligonucleotides 15 non-specifically to prevent non-specific binding in the subsequent stages. The chamber is treated for 4 hours at 37°C with a solution of 6 X SSC and 5 X Denhardt's containing boiled sonicated salmon sperm DNA at 0.1 mg/ml. SSC is 0.15 M NaCL, 0.015 M trisodium citrate, 20 pH 6.8. 100 x Denhardt's solution is 2% Ficoll (M.W. 400,000), 2% polyvinyl pyrrolidone (M.W. 400,000), and 2% bovine serum albumin and is stored at 20°C.

b. low-stringency hybridization of sample DNA to anchor sequences: Denatured sample DNA, in the 25 prehybridization solution (6 X SSC and 5 X Denhardt's containing salmon sperm DNA), and additionally containing dextran sulfate at a concentration of 100 mg/ml, is allowed to hybridize to the anchor sequence for 16 hours at 37°C.

30 c. high-stringency washing to remove non-specifically bound sample DNA: The chamber is washed three times with SSC at 4°C and twice for 30 minutes with 6 X SSC at 4°C. The chamber is then washed three times with Me<sub>4</sub>NCl wash solution at 37°C. 35 Me<sub>4</sub>NCl wash solution is 3.0 M Me<sub>4</sub>NCl, 0.05 M Tris-HCl pH 8.0, 0.002 M EDTA and sodium dodecyl sulfate at 1

mg/ml. The chamber is finally washed twice for 30 minutes in the Me<sub>4</sub>NCl wash solution at 4°C below the dissociation temperature, Td, corresponding to the length of the anchor oligonucleotide. The Td values 5 for oligonucleotides of various lengths are given by Wood et al. in Proc. Natl. Acad. Sci. (US) 82:1585 (1985), and are approximately:

	<u>PROBE LENGTH</u>	<u>Td (°C)</u>
10	11	45
	13	47
	15	49
	20	59
	30	69

15 The Td for other lengths are estimated by interpolation or extrapolation.

If one arm of the sample DNA is to be removed through use of a restriction endonuclease, the chamber is washed with a buffer compatible with the enzyme, and 20 the enzyme is added. After an appropriate time of incubation the chamber is washed with two times with the hybridization buffer used in stage b.

d. low-stringency hybridization of oligonucleotide probes to sample DNA: The 25 hybridization solution containing probes is added to the chamber and hybridization is carried out as in step b, except that the temperature is reduced to 20°C when the probe sequence is less than 15 nucleotides long.

e. high-stringency washing to remove non-specifically bound probe oligonucleotides: The 30 procedure is carried out as in step c, except that the temperature is appropriate to the length of the probe oligonucleotide as described above.

Analysis procedure

After all steps of the hybridization are carried out, the chamber is washed three times with SSC, filled with SSC and placed on the stage of an epifluorescence microscope. A 10 volt potential is applied across the two gold electrodes, with the anchor sequence-containing electrode being negative. After 2 minutes, fluorescent beads are located at low magnification (field of view 1 mm) by inspection. The magnification is increased so that the field of view is 200 microns, and a photograph is taken. The magnification is increased so that the field of view is 20 microns, and 10 successive photographs are taken starting at the anchor surface and progressing to the positive electrode, in order to obtain a panorama of the probe beads hybridized to the extended sample DNA chains.

## EXAMPLE 2

20        *Mapping Alleles of the Cystic Fibrosis Transport Regulator (CFTR) Protein Gene*

The sequence of the mRNA coding for the CFTR protein has been reported by Riordan et al., *Science*, 245:1066 (1989), and considerable information has been obtained regarding the gene itself. The mRNA is 6.2 kb long and codes for a protein of 1480 amino acids. The gene is 230 kb in length, and contains 27 exons.

Segments of the genomic sequence totaling 22.7 kb have also been obtained by Zielenski et al., *Genomics*, 10:214 (1991). The emphasis in the Zielenski study was on the boundaries of the exons since mutations in those regions frequently result in aberrant splicing and non-functional protein. A 640 nucleotide segment of the genome about 4.4 kb upstream of the start of the CFTR gene has been reported by Jones and Winistorfer, *Nucleic Acid Res.* 20:595 (1992), Genebank number

L06116. That portion of the genome is used to hybridize to an anchor sequence for further analysis of the gene in this study.

The nucleotide sequence reported by Jones and  
5 Winistorfer contains an EcoRI site towards the end proximal to the CFTR gene. The last 31 nucleotide portion of that sequence is illustrated below, with the EcoRI site underlined (a space is inserted every tenth nucleotide for reference):

10

5' tgaattcaaa ggaaaacata agatgcaatt c 3' (sequence ID 1)

3' acttaagttt ccttttgtat tctacgttaa g 5' (sequence ID 2)

15

Sequence 2 is prepared with an amino linker on the cytosine at position 12 as described in Example 1, and is used as an anchor sequence to obtain fragments of genomic DNA containing the CFTR gene. The anchor  
20 sequence is attached to the support by Method B as described in Example 1. Hybridization is performed as described in Example 1, using 66°C as the temperature for the final wash following hybridization with the following exception. Following hybridization, 5 units  
25 of EcoRI (Sigma Chemical Co.) in 100 µl of buffer (100 mM Tris HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 100 µg/ml BSA) is added to the chamber and incubated at 37°C for 60 minutes. The wash step is then repeated. After  
30 the incubation with EcoRI (the enzyme cuts between g and a on both strands at the underlined site), the portion of the sample DNA upstream of the CFTR (that is not wanted), is bound to the anchor by only two base pairs, and is thus eliminated by the subsequent washing steps.  
35 A 12 nucleotide probe specific to the three nucleotide deletion mutation delta F508, (aac acc gat

gat is made (the delta F508 sequence given in Riordan et al., *Science*, 245:1066 (1989)). The first half of this probe can hybridize to sample DNA not containing the delta F508 mutation at either the 6 nucleotides 5 coding for the isoleucine-isoleucine sequence 5' to the 508 deletion, or to the 6 nucleotide sequence coding for glycine-valine 3' to the 508 deletion. However, all 12 nucleotides can hybridize to sample DNA having the delta F508 mutation. The probe is labeled with red 10 fluorescent beads.

A 12 nucleotide probe specific to the one nucleotide deletion mutant CF3821delT (White et al., *Genomics* 10:266 (1991)),

gaa gga atg ttc, (sequence ID 3)

15 is made and labeled with green fluorescent beads as described in Example 1. This probe can only hybridize to 7 nucleotides of sample DNA that do not contain this mutation.

The probe is used to screen the sample DNA for 20 these specific CFTR mutations from a blood sample to be tested for the mutations. DNA is obtained from a blood sample as described in Example 1. The test procedure is performed as described above. Following analysis by determining the presence of the appropriate 25 colored fluorochromes, the sample is shown to contain DNA that has the delta F508 mutation.

#### EXAMPLE 3

##### *Mapping*

30 The following example illustrates determining the nucleotide sequence of a segment of a long sample DNA by walking the chromosome.

The sample oligonucleotide sequence is human genomic DNA prepared from a blood sample as described 35 in Example 1. An anchor sequence having sequence no. 1 is prepared as described in Example 2. The sample

DNA is hybridized to the anchor sequence as described in Example 2, and then incubated with EcoRI restriction endonuclease to remove one arm of the sample DNA molecule. The remaining arm is hybridized to the 5 following seven probes. The sequences for the probes is obtained from a table of random numbers. The number following each sequence is the sequence identification number (sequence IDs 4-10).

10	probe sequence	fluorescent dye	identifier
	ttgaatt (4)	red	1
	gttgcaa (5)	green	2
	cggctcg (6)	blue	3
	cggactg (7)	red and green	4
15	gatagga (8)	red and blue	5
	atgggag (9)	green and blue	6
	aggaacg (10)	red, green and blue	7

Each species of probe oligonucleotide is attached 20 to a bead containing one of three fluorescent dyes, or a combination of dyes, so that each of the seven probes is distinguishable by examination in the fluorescent microscope using different excitation wavelengths (an arbitrary identifier number from 1 to 7 is used to 25 refer to the seven species of probes in the above table). The images obtained are digitized and analyzed by computer in order to identify the dye combination as described by Ried et al., *Proc. Natl. Acad. Sci. USA* 89:1388 (1992).  
30 At low magnification a field of view is selected that contains at least 4 sample molecules (which are seen only by the fluorescent probes that have hybridized to them). The magnification is increased to obtain a field of view of 20 X 20  $\mu$ , and eight 35 overlapping images are obtained starting at the anchor surface and extending to 120  $\mu$  past the anchor toward

the opposite electrode. The X-Y positions of the probes are determined by the image analysis portion of the computer program. Since the hybridization efficiency is not 100 percent, not all sites on all 5 molecules are occupied by probes. However since the image is a composite of at least 4 sample molecules, all the hybridization sites can be identified.

Most of the segments between the probes can be selectively amplified using the polymerase chain 10 reaction (PCR) using the following method. Each probe species hybridizes to a site on the sample DNA occurring on average every 16,000 nucleotides, or an average of 12.5 sites on the 200,000 nucleotides that are examined. The total sites for all seven probe 15 species is 88 sites, and the average distance between sites is 2,300 nucleotides. There are 49 species of DNA segments between probes, one for each of the possible combinations of the 7 probes. Each of these 49 segment species can be selectively amplified using 20 the sample DNA as the template, the anchor distal probe as one primer, and the complement of the anchor proximal probe as the other primer. On average there are about two of each of the 49 species of segments on any sample DNA sequence of about 200,000 nucleotides, 25 and in the majority of cases these two segments have sufficiently different lengths that they can be separated by PAGE. The nucleotide sequence can then be determined by any of the standard methods.

Selective amplification of a segment toward the 30 distal end of the sample DNA allows preparation of a new anchor oligonucleotide which allows examination of another 200,000 nucleotide sequence of the genomic DNA distal to the first sample DNA. Using the image of the sample DNA, a segment is chosen that is less than 2,000 35 nucleotides long, is distinct in length from the other segments of that species, and is toward the anchor

distal portion of the sample DNA. This procedure is implemented in the following section.

Two probe 3-6 segments are observed on the sample DNA (probe 3 being closest to the anchor), one segment 5 close to the distal end of the sample was 1,500 nucleotides long, and the other was closer to the anchor and was 3,000 nucleotides long. The sample DNA is removed from the anchor by heating the chamber at 70°C for 3 minutes, and the 3-6 segments were amplified 10 by PCR in the standard manner. The desired 1.5 kb product is isolated using polyacrylamide gel electrophoresis (PAGE) with an acrylamide concentration of 3.5 percent. The nucleotide sequence of the 1.5 kb product is determined. The 1.5 kb product also 15 contains an EcoRI restriction site. An anchor sequence containing the site at the 5' end of the anchor is prepared and the procedure is repeated. This cycle can in principle be repeated until the telomere is reached. Such characterization of a long stretch of DNA by 20 successive steps has been called chromosome walking (see for example pg 131 of *Biochemistry*, L. Stryer, 3rd ed., W. H. Freeman and Company, New York, 1988).

#### EXAMPLE 4

25           *Preparation of Reagents and Device*  
Construction and use of electrophoresis chamber

A DNA analysis device of this invention was prepared as described below. A DNA detection chamber (or electrophoresis chamber) constructed from a square 30 frame of polyacrylamide gel (gel frame) sandwiched between two glass microscope coverslips. The bottom of the device was a generally planar support comprising a 50 X 75 mm coverslip (large coverslip) which provided support for two wire electrodes and buffer connecting 35 the electrodes to the edge of the gel frame. A cavity formed in the gel as described below was sealed by a

cover which was a 20 X 20 mm coverslip.

To prepare the electrophoresis chamber, a large cover slip, 50 X 75 mm, was immersed in acid dichromate cleaning solution overnight. After thorough washing in  
5 water and drying, one surface was treated with 10 M NaOH for 10 minutes at room temperature and again washed with water and dried. This surface was then treated with 3-meth acryloxy propyl trimethoxy silane (Polysciences, Inc., Warrington, Pennsylvania) for 10  
10 minutes at room temperature and again washed with water and dried.

A 1 mm thick slab of polyacrylamide gel was formed on top of this coverslip by constructing a chamber closed at two ends with pieces of a glass microscope  
15 slide 1 mm thick, and another piece of a glass slide on top. A solution containing 20 percent acrylamide, 1 percent bis-acrylamide, 1 percent by volume of a 10 percent sodium persulfate solution, and 0.1 percent by volume of TEMED (N, N, N', N'-tetramethyl-  
20 ethylenediamine), (Sigma Chemical Co., St. Louis, Missouri), was introduced into the chamber. (All solutions are given in weight percent (gm per 100 ml) unless otherwise specified.) Exposure of the glass surface to 3-meth acryloxy propyl trimethoxy silane  
25 covalently linked this monomer to the glass surface, and during subsequent polymerization of the gel slab this acrylamide monomer was co-polymerized into the polyacrylamide gel, thus linking it to the glass. A tight bond between the bottom of the gel and the glass  
30 surface was important to the overall method, since it prevented electro-osmotic flow under the gel.

Approximately 60 minutes was allowed for polymerization, and the side and top glass pieces were removed and the gel slab was trimmed with a razor blade  
35 to a 10 X 10 mm square. A 5 X 5 mm square was cut and removed from the middle of the gel, forming a cavity

which functioned as the electrophoresis chamber. An electrophoresis solution containing the hybrid structures to be observed (preparation of the hybrid structures is described below) was introduced into the 5 chamber and a small (20 X 20 mm) cover slip was placed on top, sealing the chamber.

A support for the DNA analysis device was constructed from a 60 X 100 mm piece of 3 mm thick transparent LUCITE sheet with a 30 X 30 mm square hole 10 cut in the middle. Since the sheet functioned only to support the device and to anchor the electrodes, the support can be of any material. The sheet material can be transparent or opaque and is preferably dielectric. However, since the support which forms the 15 electrophoresis chamber is an insulator, the sheet material can be conductive. The hole was cut in the center to permit use of an inverted epifluorescent microscope (which has an objective on the bottom of the microscope). A hole in the support is not needed for a 20 conventional microscope. Two 22 gauge nichrome wire electrodes, approximately 50 mm long were attached to one of the long sides of the Lucite by two brass bolts approximately 50 mm apart. (22 gauge wire has a 0.32 mm diameter.) The wires extended, parallel to each 25 other, over the surface of the LUCITE sheet toward the opposite edge. The chamber assembly was placed on the sheet and slipped under the wire electrodes, with the electrophoresis chamber between the electrodes and over the square hole in the sheet.

30 Several milliliters of buffer was applied to the surface of the large coverslip between each electrode and the nearby chamber edge to create a pool of liquid connecting the electrode and the gel edge. The two brass bolts were connected by flexible wires to a DPTT 35 (double pole, triple throw) switch by the side of the microscope, and the switch was connected to an

electrophoresis power supply. The power supply was turned on before observation, and the voltage adjusted to the desired value, typically between 20 and 50 volts. The DPTT switch was connected so that the 5 observer could apply the voltage in either polarity or turn it off, while looking through the microscope. Typically 50 volts was used, which resulted in a current of 5 ma passing through the chamber.

During prolonged observation, significant water 10 evaporated from the pools of buffer and several drops of distilled water were added to restore the volume. Gas evolved from the electrodes, but did not disturb the chamber as long as there was about 10 mm between the electrodes and the chamber. Observation times of 15 up to 30 minutes were performed. Chemical decomposition of the nichrome wires was observed and platinum wires, although more expensive, are preferred for long term use.

A Ziess inverted fluorescent microscope was used 20 to observe the hybrid structures. The square hole in the Lucite allowed the objective to closely approach the bottom of the chamber. Typically a 40X objective was used, which had a working distance of about 0.35 mm. Since the bottom coverslip was 0.20 mm thick, the 25 bottom 0.15 mm (150 microns) of the chamber could be observed.

Photographs were taken with an Olympus 35 mm camera. An intermediate lens with a magnification of 3.2, in combination with the 40X objective, produced a 30 total magnification on the film of 128X. One standard frame on 35 mm film is 24 X 36 mm, which thus represented a visual field of 188 X 281 microns in the electrophoresis chamber. The depth of field with this objective was about 2 microns.

35 Kodak color print film with a speed of ASA 1000 was used, and an exposure of 1/16 th of a second was

found to produce optimal results using the standard Ziess high pressure Hg arc lamp. The images on the film were transferred to a Kodak Photo CD and were read and analyzed using an Macintosh IIIfx computer (Apple Computer, Inc., Cupertino, California) and the Photoshop software package (Adobe Systems, Inc., Mountain View, California).

Preparation of fluorescent probes

10 Polystyrene beads 0.5 microns in diameter, containing internal green fluorescent dye and surface carboxyl groups were purchased from Duke Scientific Corp., Palo Alto, California. One ml of the 1 percent solid suspension supplied by the manufacturer was mixed  
15 with 300  $\mu$ l of a solution containing 5 mg/ml EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) and 1 mg/ml of sulfo-NHS (sulfo-N-hydroxy-succinimide, both purchased from Pierce Chemical Co., Rockford, Illinois) in 0.1 M MOPS (4-morpholine propane sulfonic acid, Sigma Chemical Co., St. Louis, Missouri) at pH 7.5. To this mixture was added 60  $\mu$ l 1 M NaCl, 15  $\mu$ l carrier DNA (degraded free acid from herring sperm, Cat. D3159, Sigma Chemical Co., St. Louis, Missouri, at 100 mg/ml brought to pH 7  
20 with NaOH), and 120  $\mu$ l water. Finally, 5  $\mu$ l of the oligonucleotide probe (0.45 mM in water) containing a 5' terminal primary amine group on a 12 carbon spacer (National Biosciences, Inc., Plymouth, Minnesota) was added. In this example the probe nucleotide sequence  
25 was:

cgc gcc ctc gta tca c (sequence ID 11)

The probe sequence is complementary to nucleotides 47,469 to 47,484 on the L strand of bacteriophage Lambda.

35 The suspension was agitated at 45°C for 2 hours on a shaker at 150 rpm. To block any unreacted groups on

the beads, 15  $\mu$ l of ethanol amine was added and incubation continued for an additional hour. The bead suspension was then centrifuged for 10 minutes in an Eppendorf microcentrifuge at 10,000 rpm. The 5 supernatant was discarded and the bead pellet was resuspended in 1.5 ml of water. Centrifugation and resuspension was repeated for an additional 4 times to ensure complete removal of any unreacted probe oligonucleotide that could compete with the bead 10 linked oligonucleotide. The final pellet was resuspended in 1.0 ml of water.

The extent of oligonucleotide-bead linkage was measured in order to optimize the conditions used in the linkage, and as quality control for the method. 15 The 3' end of the probe oligonucleotide had a free OH group and thus served as a substrate for terminal deoxynucleotidyl transferase. This enzyme was purchased from United States Biochemical Corp., (Cleveland, Ohio). Buffer and directions for end 20 labeling were supplied by the manufacturer, and were used to incorporate alpha  $^{35}$ S labeled dideoxyadenosine triphosphate (ICN Biomedicals, Inc., Irvine, California).

Size exclusion chromatography using P6 (Bio-Rad) 25 was used to separate the labeled oligonucleotide, which eluted at the void volume, from unincorporated triphosphate. In one typical linkage reaction, 13 percent of the total probe oligonucleotide was linked to beads, which corresponded to 45 oligonucleotides per 30 bead. While only one oligonucleotide is required to hybridize a probe bead to a sample DNA molecule, a larger number increases the probability that a bead will hybridize after a random collision, and thus improves the kinetic rate of the hybridization.

Preparation of anchors and hybrid structureMethod A

Preparation of anchors: Activated agarose balls, were purchased (Affi-gel-15, Bio-Rad Laboratories, 5 Hercules, California) and served as a solid phase for attachment of an anchor oligonucleotide sequence. Although the agarose balls were conveniently used, any solid phase, including the support for the electrophoresis chamber, can function as the solid 10 phase. The agarose balls were 75 - 300 microns in diameter and contained N-hydroxy-succinimide groups on a 15 atom spacer attached to the agarose. The spacer contained a positively-charged amino group which made it preferable for coupling to the negatively charged 15 anchor oligonucleotide.

To 2 ml of the 1:1 gel suspension supplied by the manufacturer, 10 ml of cold water was added and the suspension was centrifuged at 1500 rpm in a SORVALL RT6000B centrifuge and swinging bucket rotor at 4°C for 20 2 minutes. The supernatant was discarded and the pellet was washed two more times in 10 ml of cold water. To 0.8 ml of the gel and 0.1 ml of water remaining from the wash, 0.2 ml of 0.5 M MOPS, pH 7.5, 25 10 µl carrier DNA, and 40 µl of a 0.5 mM solution of the anchor oligonucleotide sequence containing a primary amine at the 3' terminus (National Biosciences, Inc., Plymouth, MN) in water were added. In this example the anchor oligonucleotide sequence was:

cgc gag gtc gcc gcc c (Sequence ID 12)

30 which sequence is complementary to nucleotides 1 - 16 of the L strand of bacteriophage Lambda.

The suspension was mixed gently for 2 hours at 25°C. In order to block all activated groups that had not reacted with the anchor oligonucleotide sequence, 35 10 µl of ethanol amine was added and incubation continued for an additional 1 hour. The suspension was

then washed 5 times in 20 ml of SSC in order to remove any traces of unreacted oligonucleotide that might compete with the bound oligonucleotide for a sample DNA sequence. The suspension was stored at 4°C as a 1:1  
5 slurry in SSC (standard sodium citrate, 0.15 M NaCl and 0.015 M sodium citrate at pH 7.0) and 0.04 % sodium azide.

Since the anchor oligonucleotide sequence did not have a free 3' OH group, the sequence cannot be labeled  
10 using terminal deoxynucleotidyl transferase as was done to measure the extent of linkage of probe oligonucleotides to beads. Thus, an oligonucleotide complementary to the anchor oligonucleotide, and with a free 3' OH group, was purchased and labeled as  
15 described for the probe oligonucleotide. This labeled complementary oligonucleotide was then used to measure the capacity of agarose anchors to hybridize the complementary oligonucleotide.

Aliquots of agarose anchors were hybridized in  
20 mixtures containing the same amount of radioactive complementary oligonucleotide and increasing amounts of non-radioactive complementary oligonucleotide. The total amount of complementary nucleotide that results in half maximal isotope hybridization is a measure of  
25 anchor capacity. Ten  $\mu$ l of packed agarose from one typical preparation required 700 pico moles of oligonucleotide to depress hybridization 50 percent.

Preparation of hybrid structures: One  $\mu$ l of 20X  
30 SSC was added to 20  $\mu$ l of anchor agarose slurry, the suspension centrifuged briefly and 11  $\mu$ l of the supernatant discarded. To the remaining suspension were added 3  $\mu$ l of carrier mix, consisting of 10 parts of a 10% SDS (sodium dodecyl sulfate) solution to 1  
35 part carrier DNA, 10  $\mu$ l of a freshly sonicated probe bead suspension, 3  $\mu$ l of formamide, and 2  $\mu$ l of

denatured sample DNA at a concentration of 10 µg/ml. Sample DNA (United States Biochemical Corp., Cleveland OH) had been purified from bacteriophage Lambda and was denatured by heating to 100°C for 3 minutes. The 5 resulting suspension was gently mixed and incubated at 37°C for 20 hours in capped polyethylene tubes in order to allow hybridization to occur. The tubes, with an internal volume of about 70 µl, were made by cutting the bottom 10 mm from 50 mm long, 0.4 ml centrifuge 10 tubes.

Purification of hybrid structures: The great majority of the probe beads were not expected to be hybridized to the sample DNA. Sample DNA hybridized to 15 an anchor sequence and to probe DNA which was bound to the agarose balls was purified from excess beads to remove non-hybridized probe DNA. The hybrid structures were expected to be sensitive to hydrodynamic shear, and thus the purification was designed to reduce shear 20 to a minimum.

The body of a 10 ml disposable syringe was fitted with a Lour lock stopcock (Bio-Rad Laboratories) and a 0.4 ml polyethylene centrifuge tube was press fitted on the end of the stopcock. Two ml aliquots of five 25 solutions of decreasing concentrations of sucrose (25, 20, 15, 10, and 5 percent) in 1/5 X SSC were introduced into the syringe, which was held by a clamp in a vertical position with the centrifuge tube on the bottom. A 1 mm diameter capillary tube was inserted 30 into the syringe from the top, through the open stopcock into the bottom of the centrifuge tube, and the 25 percent sucrose solution introduced. The air was thus displaced as the solution was added and bubbles could be removed by rapid pulsation of the 35 fluid. The remaining 4 solutions, in order of decreasing concentration, were gently layered at the

top of the fluid in the syringe.

A section of the end of a 28 gauge hypodermic needle was bend at 45 degrees and this section forced through the excess plastic at the tip of the tube 5 containing the hybrid structures. The tube cap was removed and by holding the needle, the tube, open end first, was immersed in the sucrose solution at the top of the syringe which was inclined at a 45 degree angle. The needle was held in place with a small cardboard 10 square having a notch cut at one edge, and the syringe was slowly returned to its original vertical position. The agarose balls fell out of the tube and down the sucrose gradient through the stopcock and into the centrifuge tube. This process required about 60 15 minutes.

The polystyrene beads have a lower density than the bottom layer of sucrose, and also sediment at less than 1/10,000 the speed of the agarose balls. Thus, 20 beads that were not attached to agarose balls remained at the top of the gradient. After the agarose balls had settled to the bottom of the tube, the stopcock was shut and the tube removed from the syringe.

.     Observation of hybrid structures: Using the 25 electrophoresis chamber, numerous hybrid structures with the characteristics expected for a sample DNA molecule hybridized at one end to the anchor sequence and the other end to the probe sequence were observed. The defining property was the extension of the bead to 30 a fixed length when the electric field was applied, followed by a slow relaxation of the bead to its original position when the field was turned off. In addition, structures were seen with many beads tangled together which also extended and relaxed in the 35 electric field. However, these tangled structures were easily distinguished from the ones representing single

DNA molecules.

One structure was chosen for quantitative measurement. Six pairs of photographs were taken, the first member of each with the electric field off and 5 the second after the field had been turned on for approximately 15 seconds. The moveable end of this structure was associated with two beads that were apparently tangled with each other, separated by 2 to 4 microns. The average movement of the two beads after 10 the field was applied is illustrated below:

<u>Film frame</u>	<u>Movement (microns)</u>
9 to 10	33.9
11 to 12	37.3

15 The linear extension of single stranded DNA and, in particular, the linear extension per nucleotide in an electric field, has not been reported in the literature. (Only double stranded DNA, which is more 20 compact, has been observed. However, one can estimate an extension of 0.6 to 0.7 nm per nucleotide. Thus, one would expect an extension of  $0.6 \times 47,484$  to  $0.7 \times 47,484$  or 28.5 to 33.2 microns. The observed 25 extensions were compatible with the values observed in this study within the uncertainties of measurement and DNA structure.

#### Method B

30 Preparation of anchors: A 100:3:1 mixture of acrylamide, bis-acrylamide (Sigma Chemical Co., St. Louis, Missouri), and 2-acrylamide glycolic acid (Aldrich Chemical Co. Inc., Milwaukee, Wisconsin), at a total concentration of 200 mg/ml, was prepared in NME buffer (0.1 M NaCl, 0.01 M MOPS pH 7.5, 0.001 M EDTA) 35 and polymerized as in Method A to form 1 X 2 X 4 mm gel bars. Anchor oligonucleotides containing a 3' terminal

primary amino group were linked to the carboxyl groups on the bars of the acrylamide copolymer using EDC and sulfo-NHS as described for the preparation of oligonucleotide fluorescent probes. The bars were  
5 washed for 4 hours with 1 ml of NME buffer per bar to remove unlinked oligonucleotide. This wash was repeated 3 times and the bars were stored at 4°C in NME buffer.

10       Preparation and observation of hybrid structures:  
One anchor bar was immersed in 100 µl of denatured sample DNA (40 µg/ml in NME buffer, heated to 100°C for 3 minutes) and incubated at 37°C for one hour to allow hybridization. The bar was then washed gently 4X with  
15 1.5 ml aliquots of NME buffer to remove unhybridized DNA, in particular, the strand of DNA not complementary to the anchor sequence which can hybridize to and then displace a strand of DNA hybridized to the anchor. The anchor bar was then placed into the electrophoresis  
20 chamber, and the chamber was filled with a 1 to 10 dilution of oligonucleotide fluorescent probe in NME buffer.

Using the microscope, a flat portion at the edge of the anchor bar was located, and an electric  
25 potential of 20 V (- to + toward the surface) was applied for 30 seconds. Fluorescent probe beads in a zone extending approximately 300 microns out from the anchor bar were transported to the surface of the anchor during this time. The electric field was turned  
30 off, and the layer of beads that had accumulated diffused away from the surface. At 20 seconds the layer of beads was about 10 microns thick, and the field was turned on for 1 second, which again compressed the layer of beads against the anchor  
35 surface.

The off for 20, on for 1 second, cycle was

repeated for a total time of 5 minutes. The electric potential was then reversed and the great majority of beads moved away from the anchor surface. After 10 seconds, a zone at the edge of the anchor surface had  
5 been cleared sufficiently of beads so that it was possible to see single beads. Clusters of beads were seen that extended out from the anchor surface by more than 10 microns, and which beads fell back on the surface when the field was turned off. These  
10 structures were consistent with probe beads tangled in sample DNA molecules.

In this method the electric field was first used to concentrate probe beads approximately 30-fold to facilitate hybridization to sample DNA at the anchor  
15 surface. The electric field was then used to remove unhybridized probe beads from the vicinity of the anchor surface to facilitate observation of hybrid structures. The probe beads move toward the + electrode in an electric field primarily because of the  
20 negative charge of about 100,000 from the persulfate groups used to initiate polymerization of the styrene. The electric field can also be used to concentrate sample DNA at the anchor surface, since the sample DNA molecule has a negative charge of about 50,000 but a  
25 hydrodynamic diameter of about 0.1 micron, 1/5th the diameter of the beads.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: Konrad, Michael W.

10

(ii) TITLE OF INVENTION: OPTICAL DETECTION OF POSITION OF  
OLIGONUCLEOTIDES ON LARGE DNA MOLECULES

15

(iii) NUMBER OF SEQUENCES: 12

20

(iv) CORRESPONDENCE ADDRESS:

15

(A) ADDRESSEE: Laura Terlizzi  
(B) STREET: 25 Metro Drive, Suite 700  
(C) CITY: San Jose  
(D) STATE: CA  
(E) COUNTRY: USA  
(F) ZIP: 95110

20

(v) COMPUTER READABLE FORM:

25

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

30

(vi) CURRENT APPLICATION DATA:

35

(A) APPLICATION NUMBER: US PCT/US94/09764  
(B) FILING DATE: 08-SEP-1994  
(C) CLASSIFICATION:

35

(viii) ATTORNEY/AGENT INFORMATION:

40

(A) NAME: Terlizzi, Laura  
(B) REGISTRATION NUMBER: 31,307  
(C) REFERENCE/DOCKET NUMBER: M-2606-1P PCT

40

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 408-283-1222

(B) TELEFAX: 408-283-1233

## (2) INFORMATION FOR SEQ ID NO:1:

45

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

55

TGAATTCAAA GGAAAACATA AGATGCAATT C

31

## (2) INFORMATION FOR SEQ ID NO:2:

60

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

65

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACTTAAGTTT CCTTTTGAT TCTACGTTAA G

31

5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAGGAATGT TC

12

20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTGAATT

7

35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTGCAA

7

50

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGGCTCG

7

65

(2) INFORMATION FOR SEQ ID NO:7:

**CGGACTG**

(2) INFORMATION FOR SEQ ID NO:8:

20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

**GATAGGA**

7

(2) INFORMATION FOR SEQ ID NO:9:

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

**ATGGGAG**

7

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10.

AGGAACCG

2

(2) INFORMATION FOR SEQ ID NO:11.

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

5 CGCGCCCTCG TATCAC

16

(2) INFORMATION FOR SEQ ID NO:12:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

20 CGCGAGGTCTG CCGCCC

16

## WHAT IS CLAIMED IS:

1. A method for analyzing a sample oligonucleotide sequence comprising:
  - 5 a. contacting a said sample oligonucleotide sequence with an anchor sequence comprising an oligonucleotide sequence which is immobilized to a support and which hybridizes with said sample oligonucleotide sequence and with a probe comprising an oligonucleotide sequence which hybridizes to a target oligonucleotide sequence to be detected in a suitable buffer to form a complex;
  - 10 b. subjecting said complex to a field which moves unbound oligonucleotide sequences away from said anchor sequence in the direction of said field; and
  - 15 c. determining whether said probe is bound to said sample oligonucleotide sequence.
- 20 2. The method of Claim 1 wherein said probe is labeled with a fluorochrome and determining whether said probe is bound to said sample oligonucleotide sequence is performed by observing the fluorochrome with a fluorescent microscope.
- 25 3. The method of Claim 2 wherein said fluorochrome is present in a bead.
4. The method of Claim 3 wherein said bead is plastic.
5. The method of Claim 4 wherein said plastic bead is 30 a polystyrene bead.
6. The method of Claim 1 wherein said anchor sequence is from about 10 to about 100 bases.
7. The method of Claim 4 wherein said anchor sequence is from about 20 to about 40 bases.
- 35 8. The method of Claim 1 wherein said field is an electric field.

9. The method of Claim 1 wherein said field is a magnetic field and a magnetic particle is attached to said sample oligonucleotide sequence or said probe.
- 5 10. The method of Claim 1 wherein said complex is subjected to said field for a time sufficient to extend said sample oligonucleotide sequence and the position of said probe in relation to said anchor sequence is also determined.
- 10 11. The method of Claim 1 additionally comprising subjecting the probe to a field which concentrates the probe near the anchor sequence during step (a).
12. The method of Claim 1 wherein said probe is from about 4 to about 100 bases.
- 15 13. A method for determining whether a first and a second target oligonucleotide sequence are on a molecule of sample DNA comprising:
  - a. contacting said sample DNA with an anchor sequence comprising an oligonucleotide sequence which is immobilized to a support and which hybridizes with said first target oligonucleotide sequence and with a probe which hybridizes with said second target oligonucleotide sequence in a suitable buffer to form a complex;
  - b. subjecting said complex to an electric field which moves unbound oligonucleotide sequences away from said anchor sequence in the direction of said field; and
  - c. determining whether said probe is bound to said sample DNA.
- 20 14. The method of Claim 13 wherein said sample DNA is not more than about 200 kilobases.
- 25 15. The method of Claim 13 wherein said sample DNA is greater than about 200 kilobases and said sample

- DNA is prepared by lysing cells in a gel and adding a fragment of said gel to said support.
16. The method of Claim 13 wherein said probe is from about 5 to about 15 bases.
- 5 17. The method of Claim 13 wherein said electric field extends said sample DNA in the direction of said field and the distance between said first and second oligonucleotide sequences is determined by determining the distance between said anchor
- 10 sequence and said probe.
18. The method of Claim 13 wherein hybridization of said sample DNA to said anchor sequence forms a double stranded oligonucleotide sequence having a site recognized by a restriction endonuclease and said restriction endonuclease is present on said solid phase or in said buffer.
- 15
19. A method for obtaining a pattern characteristic of sample DNA comprising:
- 20 a. contacting said sample DNA with an anchor sequence comprising an immobilized, conserved oligonucleotide sequence which hybridizes with said sample DNA and with a plurality of probes, each of said probes comprising an oligonucleotide sequence of from about 5 to about 15 bases in a suitable buffer to form a complex;
- 25 b. subjecting said complex to an electric field which moves unbound oligonucleotide sequences away from said anchor sequence and extends said sample DNA in the direction of said field; and
- 30 c. determining the positions of said probes from said anchor sequence.
20. The method of Claim 19 wherein said probes are from about 7 to about 10 bases.
- 35 21. The method of Claim 19 wherein said probes have

- identical oligonucleotide sequences.
22. The method of Claim 19 wherein the oligonucleotide sequence of said probe is a random sequence.
23. The method of Claim 19 wherein said probes have
- 5 different oligonucleotide sequences and are labeled with different fluorochromes.
24. A method for determining whether sample DNA and test DNA are from the same individual comprising:
- 10 a. contacting said sample DNA and said test DNA with an anchor sequence comprising an immobilized, conserved oligonucleotide sequence which hybridizes with said test DNA and with a plurality of probes, each of said probes comprising an oligonucleotide sequence of from about 5 to about 15 bases in a suitable buffer to form a complex;
- 15 b. subjecting said complex to an electric field which moves unbound oligonucleotide sequences away from said anchor sequence and extends said sample DNA in the direction of said field;
- 20 c. determining the positions of said probes from said anchor sequence for said sample DNA and said test DNA; and
- 25 d. comparing the positions of said probes for said test DNA to said probes for said sample DNA.
25. A method for determining whether a putative father is the father of a child comprising:
- 30 a. contacting said sample DNA from said putative father, said child and the mother of said child with an anchor sequence comprising an immobilized, conserved oligonucleotide sequence which hybridizes with said putative father's DNA and with a plurality of probes, each of said probes comprising an
- 35

- oligonucleotide sequence of from about 5 to  
about 15 bases in a suitable buffer to form a  
complex;
- 5           b. subjecting said complex to an electric field  
which moves unbound oligonucleotide sequences  
away from said anchor sequence and extends  
said sample DNA in the direction of said  
field;
- 10          c. determining the positions of said probes from  
said anchor sequence for said DNA from said  
putative father, said child, and said mother;
- 15          d. comparing the positions of said probes for  
said child's DNA to those of said mother; and
- 15          e. comparing any positions of the probes of said  
child which do not match with the probes of  
said mother to said probes of said putative  
father.
- 20          26. A method for determining whether a test  
oligonucleotide sequence is present in sample  
oligonucleotide sequence comprising:
- 25          a. contacting said sample DNA with an anchor  
sequence comprising an immobilized, conserved  
oligonucleotide sequence known to hybridize  
with an oligonucleotide sequence present in  
said sample oligonucleotide sequence and with  
a probe comprising an oligonucleotide  
sequence which binds to said test  
oligonucleotide sequence in a suitable buffer  
30          to form a complex;
- 35          b. subjecting said complex to an electric field  
which moves unbound oligonucleotide sequences  
away from said anchor sequence and extends  
said sample DNA in the direction of said  
field; and
- 35          c. determining whether said probe is bound to

- said sample oligonucleotide sequence.
27. The method of Claim 26 wherein said sample oligonucleotide sequence is single stranded DNA.
28. The method of Claim 26 wherein said sample oligonucleotide sequence is RNA.
- 5 29. The method of Claim 26 wherein said probe is from about 20 to about 30 bases.
30. The method of Claim 26 wherein said method additionally comprises the step of determining the 10 distance of said probe from said anchor sequence.
31. The method of Claim 26 wherein a plurality of probes which bind to a plurality of different test oligonucleotide sequences are used.
32. The method of Claim 31 wherein said each of said 15 probes is labeled with a different label.
33. A device comprising:
- a. a support having a first end and a second end which is opposite to and removed from said first end;
  - 20 b. an oligonucleotide sequence immobilized to said support at a predetermined distance from said first end of said support, defining a region between said first end and said oligonucleotide sequence;
  - 25 c. a first electrical contact connected to the region between said first end of said support and said oligonucleotide sequence; and
  - d. a second electrical contact connected adjacent the second end of said support.
- 30 34. The device of Claim 33 wherein said device comprises a plurality of oligonucleotide sequences immobilized to said support at a predetermined distance from said first end of said support.
35. The device of Claim 34 wherein each said oligonucleotide sequence is bound to gold deposited on said support.

36. The device of Claim 34 wherein each said oligonucleotide sequence is from about 20 to about 30 bases.
37. The device of Claim 35 wherein each said 5 oligonucleotide sequence is bound to said gold through a linking group.
38. The device of Claim 37 wherein said linking group is a peptide.
39. The device of Claim 37 wherein said linking group 10 is an oligonucleotide sequence.
40. The device of Claim 33 wherein said support is glass or plastic.
41. The device of Claim 33 wherein said device 15 additionally comprises a cover which extends from the first electrode to the second electrode and covers the first and second electrodes.
42. A device comprising:
  - a. a support having a first end and a second end 20 which is opposite to and removed from said first end;
  - b. a first electrical contact connected to the region between said first end of said support and said oligonucleotide sequence;
  - c. a second electrical contact connected 25 adjacent the second end of said support;
  - d. an electrophoresis chamber on said support between said first and second electrical contacts; and
  - e. an oligonucleotide sequence immobilized to a 30 solid phase in said electrophoresis chamber.
43. The device of Claim 42 wherein the solid phase is an agarose ball.

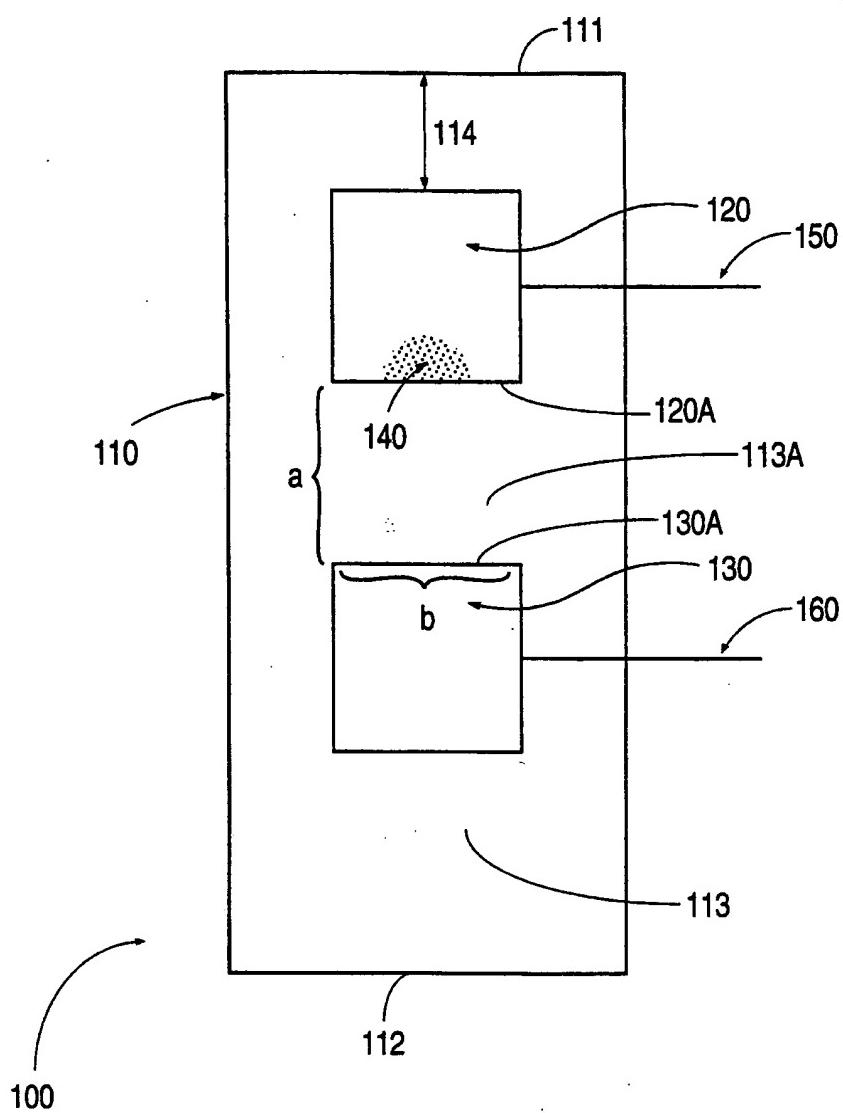


FIG. 1

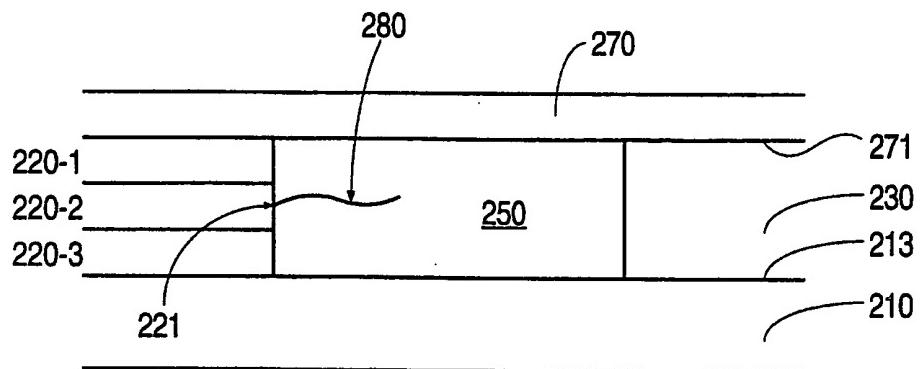
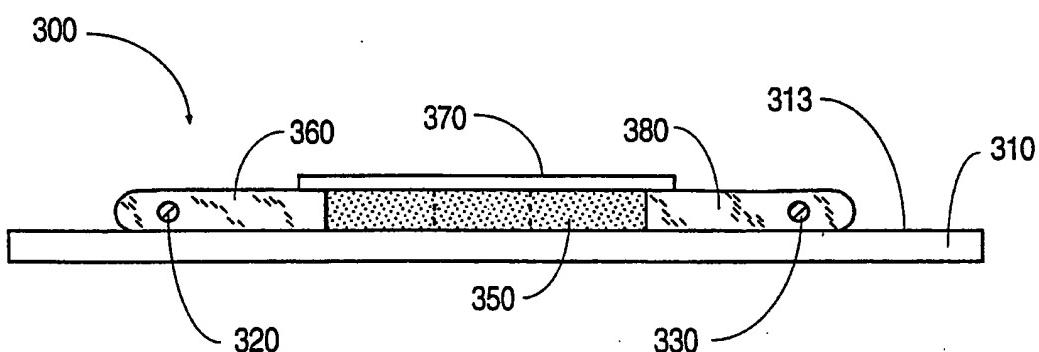
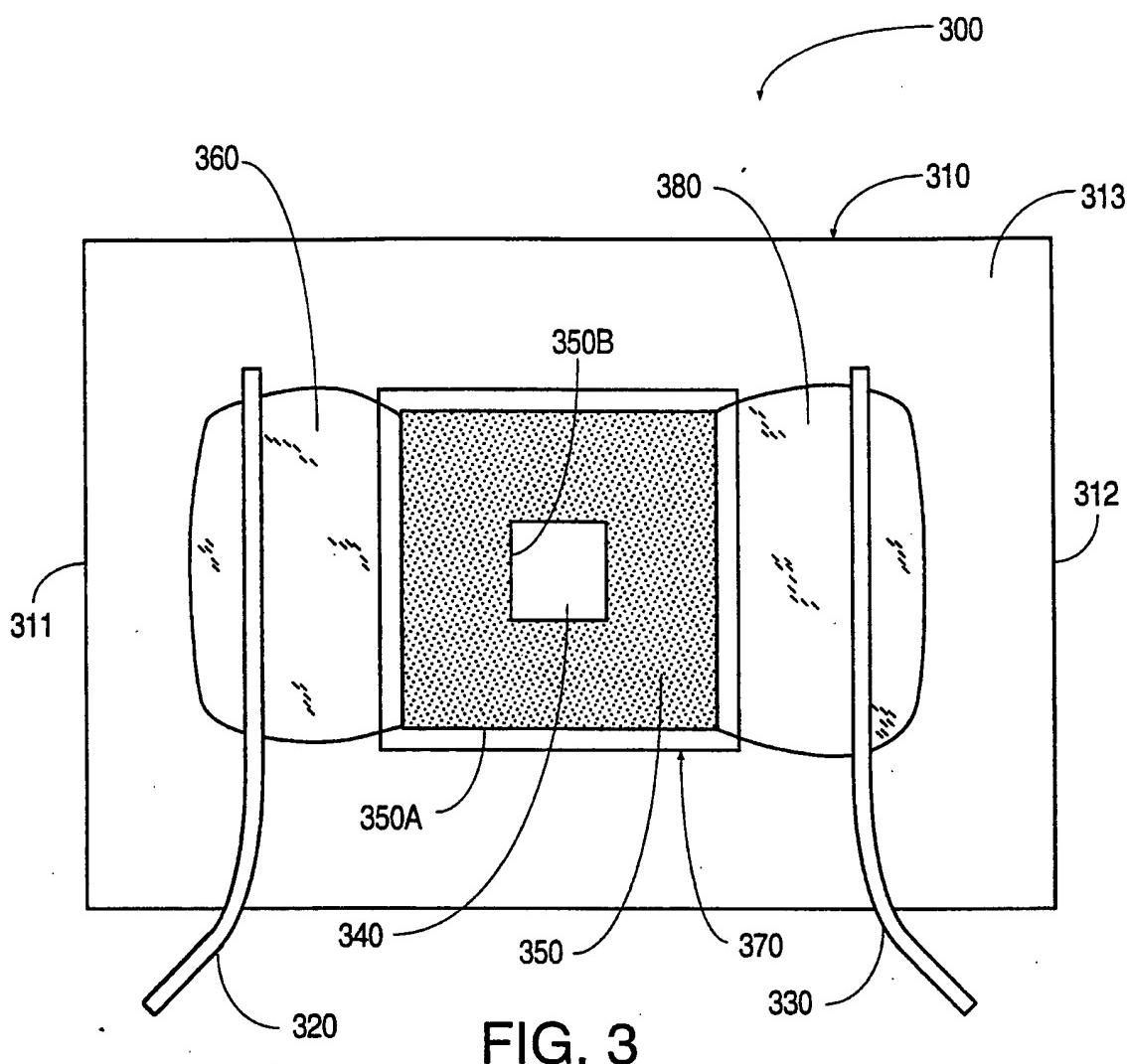


FIG. 2



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/09764

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC(6) :C12Q 1/68; G01N 27/26 US CL 435/6; 204/182.8		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
U.S. : 435/6; 422/50; 204/182.8		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,672,040 (JOSEPHSON) 09 JUNE 1987, see column 10, lines 54-63.	1-32
Y	Journal of Clinical Laboratory Analysis, Volume 3, issued 1989, Nicholls et al., "Nucleic Acid Analysis by Sandwich Hybridization", pages 122-135, see pages 126 and 128.	1-43
Y	Analytical Biochemistry, Volume 169, issued 1988, Matthews et al., "Analytical Strategies for the Use of DNA Probes", pages 1-25 , see pages 20-21.	1-43
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance    "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "E" earlier document published on or after the international filing date    "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)    "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed    "&" document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
01 DECEMBER 1994	15.12.94	
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer  STEPHANIE W. ZITOMER, PH.D. Telephone No. (703) 308-0196	

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
  - COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**